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(57) Abstract

By this invention, compositions and methods of use related to β-ketoacyl-ACP synthase, hereinafter also referred to as "synthase", are provided. Also of interest are methods and compositions of amino acid and nucleic acid sequences related to biologically active plant synthase(s). In particular, synthase protein preparations which have relatively high turnover (specific activity) are of interest for use in a variety of applications, *in vitro* and *in vivo*. Especially, protein preparations having synthase I and/or synthase II activities are contemplated hereunder. Synthase activities are distinguished by the preferential activity towards longer and shorter acyl-ACPs. Protein preparations having preferential activity towards shorter chain length acyl-ACPs are synthase I-type. Synthases having preferential activity towards longer chain length acyl-ACPs are synthase II-type. Of special interest are synthases obtainable from *Ricinus communis*.

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PLANT FATTY ACID SYNTHASES

This application is a continuation-in-part of USSN 07/568,493 filed on August 15, 1990, and a continuation-in-part of USSN 07/721,761 filed June 26, 1991.

Field of Invention

The present invention is directed to synthase enzymes relevant to fatty acid synthesis in plants, protein preparations, amino acid and nucleic acid sequences related thereto, and methods of use for such compositions.

Introduction

Background

Plant oils are used in a variety of industrial and edible uses. Novel vegetable oils compositions and/or improved means to obtain oils compositions, from biosynthetic or natural plant sources, are needed. Depending upon the intended oil use, various different fatty acid compositions are desired.

For example, in some instances having an oilseed with a higher ratio of oil to seed meal would be useful to obtain a desired oil at lower cost. This would be typical of a high value oil product. In some instances, having an oilseed with a lower ratio of oil to seed meal would be useful to lower caloric content. In other uses, edible plant oils with a higher percentage of unsaturated fatty acids are desired for cardio-vascular health reasons. And alternatively, temperate substitutes for high saturate tropical oils such as palm and coconut, would also find uses in a variety of industrial and food applications.

One means postulated to obtain such oils and/or modified fatty acid compositions is through the genetic engineering of plants. However, in order to genetically engineer plants one must have in place the means to transfer genetic material to the plant in a stable and heritable manner. Additionally, one must have nucleic acid

sequences capable of producing the desired phenotypic result, regulatory regions capable of directing the correct application of such sequences, and the like. Moreover, it should be appreciated that in order to produce a desired phenotype requires that the Fatty Acid Synthetase (FAS) pathway of the plant is modified to the extent that the ratios of reactants are modulated or changed.

Higher plants appear to synthesize fatty acids via a common metabolic pathway. In developing seeds, where fatty acids attached to triglycerides are stored as a source of energy for further germination, the FAS pathway is located in the proplastids. The first step is the formation of acetyl-ACP (acyl carrier protein) from acety-CoA and ACP catalyzed by the enzyme, acetyl-CoA:ACP transacylase (ATA). Elongation of acetyl-ACP to 16- and 18- carbon fatty acids involves the cyclical action of the following sequence of reactions: condensation with a two-carbon unit from malonyl-ACP to form a ß-ketoacyl-ACP (ß-ketoayl-ACP synthase), reduction of the keto-function to an alcohol (ß-ketoacyl-ACP reductase), dehydration to form an enoyl-ACP (ß-hydroxyacyl-ACP dehydrase), and finally reduction of the enoyl-ACP to form the elongated saturated acyl-ACP (enoyl-ACP reductase). ß-ketoacyl-ACP synthase I, catalyzes elongation up to palmitoyl-ACP (C16:0), whereas ß-ketoacyl-ACP synthase II catalyzes the final elongation to stearoyl-ACP (C18:0). Common plant unsaturated fatty acids, such as oleic, linoleic and α-linolenic acids found in storage triglycerides, originate from the desaturation of stearoyl-ACP to form oleoyl-ACP (C18:1) in a reaction catalyzed by a soluble plastid Δ-9 desaturase (also often referred to as "stearoyl-ACP desaturase"). Molecular oxygen is required for desaturation in which reduced ferredoxin serves as an electron co-donor. Additional desaturation is effected sequentially by the actions of membrane bound Δ-12 desaturase and Δ-15 desaturase. These "desaturases" thus create mono- or polyunsaturated fatty acids respectively.

A third ß-ketoacyl-ACP synthase has been reported in *S. oleracea* leaves having activity specific toward very

short acyl-ACPs. This acetoacyl-ACP synthase or "ß-ketoacyl-ACP" synthase III has a preference to acetyl-CoA over acetyl-ACP, Jaworski, J.G., *et al.*, *Plant Phys.* (1989) *90*:41-44. It has been postulated that this enzyme may be an alternate pathway to begin FAS, instead of ATA.

Obtaining nucleic acid sequences capable of producing a phenotypic result in FAS, desaturation and/or incorporation of fatty acids into a glycerol backbone to produce an oil is subject to various obstacles including but not limited to the identification of metabolic factors of interest, choice and characterization of a protein source with useful kinetic properties, purification of the protein of interest to a level which will allow for its amino acid sequencing, utilizing amino acid sequence data to obtain a nucleic acid sequence capable of use as a probe to retrieve the desired DNA sequence, and the preparation of constructs, transformation and analysis of the resulting plants.

Thus, the identification of enzyme targets and useful plant sources for nucleic acid sequences of such enzyme targets capable of modifying fatty acid compositions are needed. Ideally an enzyme target will be amenable to one or more applications alone or in combination with other nucleic acid sequences, relating to increased/decreased oil production, the ratio of saturated to unsaturated fatty acids in the fatty acid pool, and/or to novel oils compositions as a result of the modifications to the fatty acid pool. Once enzyme target(s) are identified and qualified, quantities of protein and purification protocols are needed for sequencing. Ultimately, useful nucleic acid constructs having the necessary elements to provide a phenotypic modification and plants containing such constructs are needed.

Brief Description of the Figures

Figure 1 shows the time course of inactivation of synthase activities in the first peak of synthase activity from ACP-affinity chromatography by 5 µM cerulenin. At the

outset of the experiment, the activity with C16:0-ACP is approximately twice that with C10:0-ACP, indicating an enrichment for type-II synthase in the sample. The graph shows that activity with C16:0-ACP, representing type-II synthase is virtually insensitive to cerulenin, while activity with C10:0-ACP is inhibited by greater than 80% after 10 minutes. The remaining activity with C10:0-ACP is probably low activity of type-II synthase in the sample with the C10:0-ACP substrate (approximately 10% of the activity with C16:0-ACP); this means that type-I synthase is completely inactivated at this cerulenin concentration by 15 min. (heavy dotted line).

Figure 2 provides partial amino acid sequence of peptides from the *R. communis* 50 kD β-ketoacyl ACP synthase protein listed in order found in cDNA clone. Each fragment represents amino acid sequence obtained from an HPLC purified fraction resulting from digestion of the 50 kD protein with trypsin or endoproteinase gluC. F1 shows amino terminal protein sequence obtained from the undigested 50 kD protein. A lower case x represents a sequence cycle where it was not possible to identify the amino acid residue. The positions with two amino acids represents sequences of two nearly identical peptides with a clear difference in sequence at the indicated position, suggesting microheterogeneity of the 50 kD protein.

Figure 3 provides partial amino acid sequence of peptides from the *R. communis* 46 kD β-ketoacyl ACP synthase protein. Each fragment labeled with a KR represents amino acid sequence obtained from an HPLC purified fraction resulting from digestion of the 46 kD protein with trypsin. Amino terminal protein sequence obtained from the undigested 46 kD protein is also shown (NT). "x" is as defined in Fig. 2.

Figure 4 provides the oligonucleotide primers used in PCR from the *R. communis* β-ketoacyl-ACP synthase 50 kD peptides KR4 and KR16. Primers are shown in one orientation only. Oligonucleotides in both orientations

were used as the order of peptides KR4 and KR16 in the synthase protein was not known.

Figure 5 provides cDNA and translated amino acid sequences of a 50 kD *R. communis* synthase factor B gene. Figure 5A provides preliminary cDNA sequence and the corresponding translational peptide sequence derived from the cDNA clone, pCGN2765 (2-8), which encodes the 50 kD synthase protein. The cDNA includes both the postulated transit peptide sequence (amino acids 1-42) and the sequence encoding the mature protein. Figure 5B provides the 2-8 sequence with additional 3' untranslated sequence.

Figure 6 provides amino acid sequence comparisons of the 50 kD protein sequence with other known synthase sequences. Figure 6A shows protein sequence homology of the translated amino acid sequence of the 50 kD clone with the FabB synthase gene from *E. coli*. The top line is translated amino acid sequence from the cDNA encoding the 50 kD synthase protein and the bottom line is translated amino acid sequence of the *E. coli* synthase encoded by FabB. Figure 6B shows protein sequence homology of the translated amino acid sequence of the 50 kD clone with "ORF-1" of the polyketide synthesis gene from Streptomyces. The top line is ORF-1 translated amino acid sequence and the bottom line is translated amino acid sequence from the cDNA encoding the 50 kD synthase protein.

Figure 7 provides approximately 2 kb of genomic sequence of Bce4.

Figure 8 provides a cDNA sequence and the corresponding translational peptide sequence derived from *C. tinctorius* desaturase. The cDNA includes both the plastid transit peptide sequence (amino acids 1-33) and the sequence encoding the mature protein.

Figure 9 provides preliminary partial cDNA sequence of *Brassica campestris* desaturase. Figure 4A represents partial DNA sequence of a 1.6 kb clone, pCGN3235, from the 5' end of the clone. Figure 4B represents partial DNA sequence of a 1.2 kb clone, pCGN3236, from the 5' end of the clone. Initial sequence from the 3' ends of the two

Brassica campestris desaturase clones, indicates that pCGN3236 is a shorter clone from the same gene as pCGN3235.

Figure 10 provides cDNA and translated amino acid sequences of a *R. communis* 46 kD synthase factor A gene.

Figure 11 provides cDNA and translated amino acid sequences of *Brassica* synthase factor B genes. Figure 11A provides sequences of the cDNA insert of pCGN3248. Figure 11B provides sequences of clone 4A.

Figure 12 provides a comparison of synthase amino acid sequences. "RC46" is a portion of the translated amino acid sequence of the *Ricinus communis* synthase factor A gene. "RC50" is a portion of the translated amino acid sequence of the *R. communis* synthase factor B gene. "BC50" is a portion of the translated amino acid sequence of the *Brassica* factor B gene of pCGN3248. "fabB" represents translated amino acid sequence of an *E. coli* synthase I gene (Kauppiner *et al.*, *Carlsberg Res. Commun* (1988) *53*:357-370).

Summary of the Invention

By this invention, compositions and methods of use related to ß-ketoacyl-ACP synthase, hereinafter also referred to as "synthase", are provided. Also of interest are methods and compositions of amino acid and nucleic acid sequences related to biologically active plant synthase(s).

In particular, synthase protein preparations which have relatively high turnover (specific activity) are of interest for use in a variety of applications, *in vitro* and *in vivo*. Especially, protein preparations having synthase I and/or synthase II activities are contemplated hereunder. Synthase activities are distinguished by the preferential activity towards longer and shorter acyl-ACPs. Protein preparations having preferential activity towards shorter chain length acyl-ACPs are synthase I-type. Synthases having preferential activity towards longer chain length acyl-ACPs are synthase II-type. Of special interest are synthases obtainable from *Ricinus communis*.

Nucleic acid sequences encoding a synthase biologically active in a host cell may be employed in nucleic acid constructs to modulate the amount of synthase present in the host cell, especially the relative amounts of synthase I-type and synthase II-type proteins when the host cell is a plant host cell. A synthase may be produced in host cells for harvest or as a means of effecting a contact between the synthase and its substrate. Host cells include prokaryotes and/or eukaryotes. Plant host cells containing recombinant constructs encoding a synthase, as well as plants and cells containing modified levels of synthase protein(s) are also provided.

By this invention, methods of catalyzing the condensation reaction between an acyl-ACP having a chain length of C_2 to C_{16} and malonyl-ACP is effected by contacting an acyl-ACP and malonyl-ACP substrates with a synthase obtainable from *R. communis* under conditions which permit the condensation of the reactants. Although the reaction employs a *R. communis* synthase, this reaction may occur outside of a *R. communis* cell. Using various techniques, this reaction can be conducted *in vitro* or in other plant cell hosts *in vivo*. For example, one may grow a plant cell having integrated in its genome an expression construct having, in the 5' to 3' direction of transcription, a plant expressible promoter, a DNA sequence encoding a synthase obtainable from *R. communis* and a transcription termination region. Of interest is the modulation of synthases alone and in conjunction with each other. For expression in plants, the use of promoters capable of preferentially directing transcription and translation in embryo tissue to regulate the expression of a synthase may be desired.

In addition, nucleic acid constructs may be designed to decrease expression of endogenous synthase in a plant cell as well. One example is the use of an anti-sense synthase sequence under the control of a promoter capable of expression in at least those plant cells which normally produce the enzyme.

Additionally, one may wish to coordinate expression of a synthase with the expression of other introduced sequences encoding other enzymes related to fatty acid synthesis, for example plant thioesterases, especially medium-chain thioesterases, desaturases, especially Δ-9 desaturases, and the like. When nucleic acid constructs encoding such factors are prepared for introduction into a plant cell, the transcriptional initiation regions will most likely be different from each other.

Synthase preparations obtained from *R. communis*, substantially free of protein contaminants are described. *R. communis* synthase preparations may be obtained which demonstrate synthase I-type activity and those which demonstrate synthase II-type activity. Enzyme specific activities of up to 16 μmol/min/mg protein and 1.7 μmol/min/mg protein have been observed for synthase I type and synthase II-type protein preparations, respectively. Proteins or protein preparations displaying such activities, alone or in combination, may be contacted with fatty acid synthesis substrates to drive synthase condensation reactions. The amino acid and nucleic acid sequences corresponding to the various preparations may be deduced and used to obtain other homologously related synthases.

Detailed Description of the Invention

A plant synthase of this invention includes any sequence of amino acids, polypeptide, peptide fragment or other protein preparation, whether derived in whole or in part from natural or synthetic sources which demonstrates the ability to catalyze a condensation reaction between an acyl-ACP or acyl-CoA having a chain length of C_2 to C_{16} and malonyl-ACP in a plant host cell. A plant synthase will be capable of catalyzing a synthase reaction in a plant host cell, i.e., *in vivo*, or in a plant cell-like environment, i.e., *in vitro*. Typically, a plant synthase will be derived in whole or in part from a natural plant source.

In addition, synthase from other sources such as bacteria or lower plants, may also be useful in plants and thus be considered a plant synthase in this invention. For example, the *E. coli* synthase protein encoded by the fab B gene is shown herein to have homology to plant synthase proteins.

Synthase I demonstrates preferential activity towards acyl-ACPs having shorter carbon chains, C_2-C_{14}; synthase II demonstrates preferential activity towards acyl-ACPs having longer carbon chains, C_{14}-C_{16}. Synthase III demonstrates preferential activity towards acyl-CoAs having very short carbon chains, C_2 to C_6. Other plant synthases may also find applicability by this invention, including synthase III type activities. Differences between synthases I, II, and III are also observed in inhibition with cerulenin. Synthase I is most sensitive, synthase II less sensitive and synthase III the least sensitive to cerulenin. In Fig. 1, a time course assay of cerulenin effects on synthase I and synthase II activities is provided. It can be seen from these results, that synthase II has some synthase I-type activities.

Synthases include modified amino acid sequences, such as sequences which have been mutated, truncated, increased and the like, as well as such sequences which are partially or wholly artificially synthesized. Synthases and nucleic acid sequences encoding synthases may be obtained by partial or homogenous purification of plant extracts, protein modeling, nucleic acid probes, antibody preparations, or sequence comparisons, for example. Once purified synthase is obtained, it may be used to obtain other plant synthases by contacting an antibody specific to *R. communis* synthase with a plant synthase under conditions conducive to the formation of an antigen:antibody immunocomplex and the recovery of plant synthase which reacts thereto. Once the nucleic acid sequence encoding a synthase is obtained, it may be employed in probes for further screening or used in genetic engineering constructs

for transcription or transcription and translation in host cells, especially plant host cells.

Recombinant constructs containing a nucleic acid sequence encoding a synthase and a heterologous nucleic acid sequence of interest may be prepared. By heterologous is meant any sequence which is not naturally found joined to the synthase sequence. Hence, by definition, a sequence joined to any modified synthase is not a wild-type sequence. Other examples include a synthase from one plant source which is integrated into the genome of a different plant host.

Constructs may be designed to produce synthase in either prokaryotic or eukaryotic cells. The increased expression of a synthase in a plant cell or decreased amount of endogenous synthase observed in a plant cell are of special interest. Moreover, in a nucleic acid construct for integration into a plant host genome, the synthase may be found in a "sense" or "anti-sense" orientation in relation to the direction of transcription. Thus, nucleic acids may encode biologically active synthases or sequences complementary to the sequence encoding a synthase to inhibit the production of endogenous plant synthase. By transcribing and translating a sense sequence in a plant host cell, the amount of synthase available to the plant FAS complex is increased. By transcribing or transcribing and translating an anti-sense sequence in a plant host cell, the amount of the synthase available to the plant FAS is decreased. Ideally, the anti-sense sequence is very highly homologous to the endogenous sequence. Other manners of decreasing the amount of synthase available to FAS may be employed, such as ribozymes or the screening of plant cells transformed with constructs containing sense sequences which in fact act to decrease synthase expression, within the scope of this invention. Other analogous methods may be applied by those of ordinary skill in the art.

Synthases may be used, alone or in combination, to catalyze the elongating condensation reactions of fatty

acid synthesis depending upon the desired result. For example, rate influencing synthase activity may reside in synthase I-type, synthase II-type, synthase III-type or in a combination of these enzymes.

Thus, over-expression of synthase I could serve to increase fatty acid yield, and/or the proportion of palmitic acids (C16:0) found in the system. Alternatively, as a critical enzyme in several fatty acid elongation steps, reducing endogenous synthase I might effectively provide low yields of fatty acids. As the last enzyme in the fatty acid elongation pathway, synthase II may be a critical factor to increase production of fatty acids. Increased availability of synthase II to FAS may in effect "drive" the rate of reaction forward and result in a larger pool of long chain fatty acids. In turn, the presence of an increased amount of fatty acids with 18 carbons may result ultimately, in the increased production of triglycerides. In a like manner, the decrease of synthase II may work to decrease one or both of these mechanisms. Because synthase II catalyzes final elongation steps, it may require support from other synthase factors to create the desired effect. In particular, the combined presence of synthase I and synthase II are contemplated for the generation of a high composition of oleic fatty acids and/or increased triglyceride production. In addition, the production of palmitate may be further enhanced by a combination of increased synthase I production and reduction in endogeous synthase II. Thus, various synthase factors may be combined in a like fashion to achieve desired effects.

Other applications for use of cells or plants producing synthase may also be found. For example, potential herbicidal agents selective for plant synthase may be obtained through screening to ultimately provide environmentally safe herbicide products. Especially in that bacterial systems do not have an enzyme equivalent to plant synthase II, they may be particularly useful systems for the screening of such synthase II based herbicides.

The synthase can also be used in conjunction with chloroplast lysates to enhance the production and/or modify the composition of the fatty acids prepared *in vitro*. The synthase can also be used for studying the mechanism of fatty acid formation in plants and bacteria. For these applications, constitutive promoters may find the best use.

Constructs which contain elements to provide the transcription and translation of a nucleic acid sequence of interest in a host cell are "expression cassettes". Depending upon the host, the regulatory regions will vary, including regions from structural genes from viruses, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli, B. subtilis, Saccharomyces cerevisiae*, including genes such as ß-galactosidase, T7 polymerase, trp-lac (tac), trp E and the like.

An expression cassette for expression of synthase in a plant cell will include, in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region (also known as a "promoter") functional in a plant cell, a nucleic acid sequence encoding a synthase, and a transcription termination region. Numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the desaturase structural gene. Among transcriptional initiation regions used for plants are such regions associated with cauliflower mosaic viruses (35S, 19S), and structural genes such as for nopaline synthase or mannopine synthase or napin and ACP promoters, etc. The transcription/translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons. Thus, depending upon the intended use, different promoters may be desired.

Of special interest in this invention are the use of promoters which are capable of preferentially expressing the synthase in seed tissue, in particular, at early stages of seed oil formation. Selective modification of seed fatty acid/oils composition will reduce potential adverse effects to other plant tissues. Examples of such seed-specific promoters include the region immediately 5' upstream of a napin or seed ACP genes such as described in EP 0 255 378 (published 2/3/88), desaturase genes such as described in Thompson *et al* (*Proc. Nat. Acad. Sci.* (1991) *88:2578-2582*), co-pending USSN 494,106 and Figs. 8 and 9, herein, or Bce-4 gene such as described in co-pending USSN 494,722, and Fig. 7 herein. Alternatively, the use of the 5' regulatory region associated with the plant synthase structural gene, i.e., the region immediately 5' upstream to a plant synthase structural gene and/or the transcription termination regions found immediately 3' downstream to the plant synthase structural gene, may often be desired. In general, promoters will be selected based upon their expression profile which may change given the particular application.

Sequences found in an anti-sense orientation may be found in cassettes which at least provide for transcription of the sequence encoding the synthase. By anti-sense is meant a DNA sequence in the 5' to 3' direction of transcription which encodes a sequence complementary to the sequence of interest. It is preferred that an "anti-sense synthase" be complementary to a plant synthase gene indigenous to the plant host. Any promoter capable of expression in a plant host which causes initiation of high levels of transcription in all storage tissues during seed development is sufficient. Seed specific promoters may be desired.

In addition, one may choose to provide for the transcription or transcription and translation of one or more other sequences of interest in concert with the expression or anti-sense of the synthase sequence, for example sequences encoding a plant desaturase such as

described in co-pending USSN 494,106 and USSN unassigned, filed on or about 8/13/90 entitled "Plant Desaturases - Compositions and Use", seed or leaf acyl carrier protein such as described in co-pending USSN 437,764, medium-chain plant thioesterase such as described by Pollard, *et al.*, (*Arch. Biochem. Biophys.* (1991) *284*:306-312) and in co-pending USSN 514,030, or other sequence encoding an enzyme capable of affecting plant lipids, to affect alterations in the amounts and/or composition of plant oils. The general methods of use and means to determine related sequences and compositions described above as to synthase enzymes may be applied to these enzymes as well.

One may wish to integrate nucleic acids encoding a desaturase sense sequence and synthase sense sequence into the genome of a host cell. A plant desaturase includes any enzyme capable of catalyzing the insertion of a first double band into a fatty acid-ACP moiety, especially Δ-9 desaturase. Such a combination may be designed to modify the production of unsaturated fatty acids and thus either lead to significantly lower or higher saturated fat upon the expression of both enzymes in a plant host cell. As desaturase acts upon the longer chain fatty acyl-ACPs, the resulting product of synthase II activity, various applications are possible. Of interest is the combination of an enhanced production of both synthase II and Δ-9 desaturase for the production of fatty acids having little or no completely saturated chains. It may also be of interest to provide for the increased production of synthase II and a decreased production of desaturase for the production of high stearate (C18:0) fatty acid compositions. The modified pool of saturated/unsaturated fatty acids may be reflected in the composition of resulting triglycerides. In a different embodiment, it may be desired to combine the increased expression of a synthase, such as synthase I, with a medium-chain plant thioesterase. Plants containing a medium-chain plant thioesterase, an enzyme capable of having preferential hydrolase activity toward one or more medium-chain (C8 to

C14) acyl-ACP substrates, are contemplated for the production of medium chain fatty acids, especially laurate (C12:0). In combination with an increased level of one or more synthases, these effects may be augmented.

When one wishes to provide a plant transformed for the combined effect of more than one nucleic acid sequence of interest, typically a separate nucleic acid construct will be provided for each. The constructs, as described above contain transcriptional or transcriptional and translational regulatory control regions. One skilled in the art will be able to determine regulatory sequences to provide for a desired timing and tissue specificity appropriate to the final product in accord with the above principles set forth as to synthase expression or anti-sense constructs. When two or more constructs are to be employed, whether they are both related to synthase sequences or a synthase sequence and a sequence encoding an enzyme capable of affecting plant lipids, it is desired that different regulatory sequences be employed in each cassette to reduce spontaneous homologous recombination between sequences. The constructs may be introduced into the host cells by the same or different methods, including the introduction of such a trait by crossing transgenic plants via traditional plant breeding methods, so long as the resulting product is a plant having both characteristics integrated into its genome.

Of special interest are synthases which appear to have superior kinetic properties, isolated from protein preparations obtainable from plants such as *Prunus amygdalus*, or more preferred *Ricinus communis*. As shown in Table I, in comparison of crude extracts from *Spinacia oleracea* leaf and some plants with low levels of saturated oils, *R. communis* and *P. amygdalus* show a markedly higher total activity of synthase II activity per gram fresh weight; calculated specific activities are also higher.

Table I

Synthase II Yields and Substrate Km Values in Crude Extracts From Various Sources[1]

Source Tissue	Protein mg/g Fr Wt	Activity mU/g Fr Wt	Specific Activity mU/mg Protein	Km (C16-ACP) μM
S. oleracea Leaf	7.15	6.61	0.962	14.0
B. napus Seed	7.62	6.58	0.864	22.0
R. communis Endosperm	21.0	346.0	16.5	15.7
P. amygdalus Embryo	8.07	62.3	7.73	22.4

[1]*S. oleracea* leaf extract was prepared according to Shimakata & Stumpf, PNAS 79:5808-5812 (1983). The remaining samples were prepared from ground tissue which had been mixed with two volumes of buffer (2 mg/g fresh meal weight) containing 50 mM potassium phosphate and 2mM dithiothreitol, pH 7.5, of which the supernatant fluid was collected after centrifugation. Protein was assayed by the Bradford method. (*Analy. Biochem.* (1976) 72:248-254) Synthase II was assayed as described in Example 2 and with the palmitoyl-ACP in varying concentrations.

Tests also compared the sensitivity of the crude extracts to substrate concentration. It was assumed that malonyl-ACP would be proportional to added ACP due to the presence of malonyl transacylase in the crude extracts. The results showed very little differences in K_m for palmitoyl-ACP or in sensitivity to ACP/malonyl-ACP between species. Other analogous plant sources may be determined by similar testing.

The exceptionally low level of saturated fat (<1%) and high activity of the synthase II enzyme in crude extracts are qualities which indicate *R. communis* as exemplary of a preferred enzyme source. Of special interest is *R. communis* seed produced by a primary inflourescence or

flower spike, as it usually has a higher oil content and higher synthase II specific activity, than the seed of branches from a secondary or tertiary inflourescence (secondary spikes). A major difficulty with the *R. communis* tissue is the toxicity of the *R. communis* seed storage ricins. Ricin is removed from the extract during the purification of the synthases as discussed in more detail below. Also, the stage at which the seeds are harvested influences the amount of ricin present in the tissue.

"Early" seeds have a very small central opaque core, a predominant translucent tissue, and very low levels of protein and synthase II activity. "Prime" seed tissue, which is found at about 21-28 days after flower opening have a central opaque white core, which contains the deposited seed oil and ricin, surrounded by a translucent white tissue. The seed coat is just beginning to harden and turn from white to a purplish-brown. The translucent tissue gradually disappears and the opaque core increases during maturation. As shown in Table II, the "Prime" tissue from the primary flower spikes have the highest synthase II specific activity; these are a main source of synthase II. The more mature "Late" seeds continue to exhibit high synthase II activity, but the large increase in deposition of ricin dilutes the specific activity. With improved methods of removing ricin, the older seeds may prove to be preferred sources as well.

Table II

ß-Ketoacyl-ACP Synthase II Activity in Developing Seeds on Primary and Secondary Flower Spikes of *R. communis*[1]

Sample	Specific Activity (µUnits/mg protein)
A	936 ± 39
B	2615 ± 139
C	10364 ± 544
D	9537 ± 1342
E	8386 ± 261
F	5034 ± 171
G	1735 ± 134

H	1459 ± 4
I	4936 ± 87
J	3466 ± 30
K	6115 ± 193

[1]Individual *R. communis* seeds were identified as Early (A,B,G,H), Prime (C,D,I) or Late (E,F,J,K). Seeds A-F were from primary spikes and seeds G-K were from secondary spikes. Each endosperm tissue from seed was ground in two volumes (ml/g fresh weight) of 40 mM potassium phosphate, 20% glycerol (v/v), 1 mM sodium EDTA, 2 mM DTT, pH 7.5, with a pestle in a microcentrifuge tube. The homogenate was clarified by centrifugation at 11,600 x g for 15 minutes. The supernatant was assayed for protein by the Bradford method (*Analy. Biochem.* (1976) 72:248-254) and for synthase II activity as described in Example 2.

As demonstrated more fully in the Examples, extraction and purification of synthases I and II from *R. communis* is obtained by ammonium sulfate fractionation (pH 7.5) and then subjecting the supernatant fluid to saturation with ammonium sulfate to precipitate the synthases. The pellet containing synthase activity is resuspended and the activity are bound to Reactive Green-19 Agarose. A column containing the activity bound to the Green-19 Agarose is prepared and the synthase activity is eluted in a high salt wash. The protein associated with fractions of peak activity is partially desalted and then absorbed to an ACP-Sepharose column and eluted with a gradient of 100-250 mM potassium phosphate buffer. The ACP column removes several proteins including a major contaminant which also showed a molecular weight at about 50 kD. Fractions assayed for synthase activity show that a major peak having primarily synthase II-type, but also containing some synthase I-type, activity elutes first and that fractions eluting after the major peak contain primarily synthase I-type activity.

When applied to SDS-PAGE analysis, fractions from the major peak having synthase II activity are shown to contain two major bands, one at about 46kD and a second at about 50 kD. Synthase II activity has not been observed separate from protein preparations containing both the 50 kD and a 46 kD band. The relationship between the 50 and 46 kD bands is under further investigation, including cerulinin assays and *E. coli* expression studies. Two-dimensional gel analysis separates the 50 kD band into at least two spots.

Fractions eluting after the major peak contain mainly synthase I-type activity and show one distinctive band at about 50kD. Tests with monoclonal antibodies indicate that the 50kD proteins from the two fractions are very similar. Additional work is underway to further characterize these proteins biochemically.

Given the few bands provided in the SDS-PAGE, immediate efforts to obtain the corresponding amino acid and/or nucleic acid sequences thereto are possible in accordance with methods familiar to those skilled in the art. From such sequences, synthase activity may be further confirmed with expression in controlled systems, such as *E. coli*, or by observation of effects of transcription of anti-sense nucleic acid fragments and the like.

Amino acid sequences of fragments corresponding to purified protein preparations may be obtained through digestion with a protease, such as trypsin, and sequencing of resulting peptide fragments. Amino acid sequences of peptide fragments derived from the 50 kD protein are shown in Fig. 2 and from the 46 kD protein in Fig. 3 (Fig. 4 shows peptide sequences of the 50 kD peptide used to design oligonucleotide by "reverse translation" of peptide fragment amino acid sequences). DNA sequences are chosen for use in Polymerase Chain Reactions (PCR) using *R. communis* endosperm cDNA as a template to obtain longer DNA sequences. The resulting PCR-generated sequences are then used as labeled probes in screening a *R. communis* endosperm cDNA or genomic DNA library. In this manner, the full length clones corresponding to the *R. communis* proteins seen on the SDS-PAGE may be obtained if desired. Figure 5 is a DNA sequence corresponding to the 50kD protein obtained in this manner. Other plant synthase genes may be obtained by screening of cDNA or genomic libraries from other plant sources with probes derived from the synthase cDNA.

In figures 6A and 6B we present sequence comparison between the translated amino acid sequence from an isolated cDNA clone which encodes the 50 kD synthase protein and two other known synthase proteins. Comparison to FabB, the

gene encoding ß-ketoacyl-ACP synthase I in *E. coli*. in Figure 6A, indicates there is extensive identical homology of amino acids, especially near the active site, amino acids 219-223 of the FabB protein. The 50 kD translated amino acid sequence also has significant identical homology to a polyketide synthase protein in *Streptomyces glaucescens* that is encoded by ORF-1, as shown in Figure 6B. The polyketide synthase protein is homologous to other known synthases, especially at the active site.

A DNA sequence of this invention may include genomic or cDNA sequence. A cDNA sequence may or may not contain pre-processing sequences, such as transit peptide sequences. Transit peptide sequences facilitate the delivery of the protein to a given organelle and are cleaved from the amino acid moiety upon entry into the organelle, releasing the "mature" protein (or enzyme). As synthases are part of the FAS pathway of plastid organelles, such as the chloroplast, proplastid, etc., transit peptides may be required to direct the protein(s) to substrate. A transit peptide sequence from any plastid-translocating sources may be employed, such as from ACP, especially seed ACP, small subunit of ribulose bisphosphate carboxylase (RuBC), plant desaturase or from the native sequence naturally associated with the respective synthase.

The complete genomic sequence of a plant synthase may be obtained by the screening of a genomic library with a probe and isolating those sequences which hybridize thereto as described more fully below. Regulatory sequences immediately 5', transcriptional and translational initiation regions, and 3', transcriptional and translational termination regions, to the synthase may be obtained and used with or without the synthase structural gene.

Other synthases and/or synthase nucleic sequences are obtainable from amino acid and DNA sequences provided herein. "Obtainable" refers to those plant synthases which have sufficiently similar sequence to that of the native sequence(s) of this invention to provide a biologically

active synthase. One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover synthases and/or synthase nucleic acid sequences from other sources. Thus, sequences which are homologously related to or derivations from either *R. communis* synthase I or II are considered obtainable from the present invention.

"Homologeously related" includes those nucleic acid sequences which are identical or conservatively substituted as compared to the native sequence. Typically, a homologously related nucleic acid sequence will show at least about 60% homology, and more preferably at least about 70% homology, between the *R. communis* synthase and the given plant synthase of interest, excluding any deletions which may be present. Homology is determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions.

Probes can be considerably shorter than the entire sequence, but should be at least about 10, preferably at least about 15, more preferably at least 20 or so nucleotides in length. Longer oligonucleotides are also useful, up to the full length of the gene encoding the polypeptide of interest. Both DNA and RNA can be used.

A genomic library prepared from the plant source of interest may be probed with conserved sequences from a *R. communis* synthase cDNA to identify homologously related sequences. Use of an entire *R. communis* synthase cDNA may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. In this general manner, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the synthase gene from such plant source. cDNA libraries prepared from other plant sources of interest may be screened as well, providing the coding region of synthase genes from such plant sources.

In use, probes are typically labeled in a detectable manner (for example with ^{32}P-labeled or biotinylated nucleotides) and are incubated with single-stranded DNA or RNA from the plant source in which the gene is sought, although unlabeled oligonucleotides are also useful. Hybridization is detected, typically using nitrocellulose paper or nylon membranes by means of the label after single-stranded and double-stranded (hybridized) DNA or DNA/RNA have been separated. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Thus, plant synthase genes may be isolated by various techniques from any convenient plant. Plant genes for synthases from developing seed obtained from other oilseed plants, such as *C. tinctorius* seed, rapeseed, cotton, corn, soybean cotyledons, jojoba nuts, coconut, peanuts, oil palm and the like are desired as well as from non-traditional oil sources, such as *S. oleracea* chloroplast, avocado mesocarp, *Cuphea*, California Bay and *Euglena gracillis*. Synthases, especially synthase I, obtained from *Cuphea* may show specialized activities towards medium chain fatty acids. Such synthase may be of special interest for use in conjunction with a plant medium-chain thioesterase.

Once the desired plant synthase sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized, where one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like. For expression, the open reading frame coding

for the plant synthase or functional fragment thereof will be joined at its 5' end to a transcriptional initiation regulatory control region. In some instances, such as modulation of plant synthase via a nucleic acid sequence encoding synthase in an anti-sense orientation, a transcription initiation region or transcription/translation initiation region may be used. In embodiments wherein the expression of the synthase protein is desired in a plant host, a transcription/translation initiation regulatory region, is needed. Additionally, modified promoters, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV promoters, may be employed for some applications.

As described above, of particular interest are those 5' upstream non-coding regions which are obtained from genes regulated during seed maturation, particularly those preferentially expressed in plant embryo tissue, such as ACP-and napin-derived transcription initiation control regions. Such regulatory regions are active during lipid accumulation and therefore offer potential for greater control and/or effectiveness to modify the production of plant desaturase and/or modification of the fatty acid composition. Especially of interest are transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts. For this purpose, the transcript initiation region of acyl carrier protein isolated from *B. campestris* seed and designated as "Bcg 4-4" and a gene having an unknown function isolated from *B. campestris* seed and designated as "Bce-4" are also of substantial interest.

Briefly, Bce4 is found in immature embryo tissue at least as early as 11 days after anthesis (flowering), peaking about 6 to 8 days later or 17-19 days post-anthesis, and becoming undetectable by 35 days post-anthesis. The timing of expression of the Bce4 gene closely follows that of lipid accumulation in seed tissue.

Bce4 is primarily detected in seed embryo tissue and to a lesser extent found in the seed coat. Bce4 has not been detected in other plant tissues tested, root, stem and leaves.

Bce4 transcript initiation regions will contain at least 1 kb and more preferably about 5 to about 7.5 kb of sequence immediately 5' to the Bce4 structural gene.

The Bcg 4-4 ACP message presents a similar expression profile to that of Bce4 and, therefore, also corresponds to lipid accumulation in the seed tissue. Bcg 4-4 is not found in the seed coat and may show some differences in expression level, as compared to Bce4, when the Bcg 4-4 5' non-coding sequence is used to regulate transcription or transcription and translation of a plant Δ-9 desaturase of this invention.

The napin 1-2 message is found in early seed development and thus, also offers regulatory regions which can offer preferential transcriptional regulation of a desired DNA sequence of interest such as the plant desaturase DNA sequence of this invention during lipid accumulation. Napins are one of the two classes of storage proteins synthesized in developing *Brassica* embryos (Bhatty, *et al., Can J. Biochem.* (1968) *46*:1191-1197) and have been used to direct tissue-specific expression when reintroduced into the *Brassica* genome (Radke, *et al., Theor. Appl. Genet.* (1988) *75*:685-694).

As to regulatory transcript termination regions, these may be provided by the DNA sequence encoding the plant synthase or a convenient transcription termination region derived from a different gene source, especially the transcript termination region which is naturally associated with the transcript initiation region. Typically, the transcript termination region will contain at least about 1 kb, preferably about 3 kb of sequence 3' to the structural gene from which the termination region is derived.

In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which

is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformed cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species into which the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

The manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection, electroporation, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present

in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), either being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cell and gall.

A preferred method for the use of *Agrobacterium* as the vehicle for transformation of plant cells employs a vector having a broad host range replication system, at least one T-DNA boundary and the DNA sequence or sequences of interest. Commonly used vectors include pRK2 or derivatives thereof. See, for example, Ditta *et al.*, *PNAS USA*, (1980) 77:7347-7351 and EPA 0 120 515, which are incorporated herein by reference. Normally, the vector will be free of genes coding for opines, oncogenes and *vir*-genes. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

The vector is used for introducing the DNA of interest into a plant cell by transformation into an *Agrobacterium* having *vir*-genes functional for transferring T-DNA into a plant cell. The *Agrobacterium* containing the broad host range vector construct is then used to infect plant cells under appropriate conditions for transfer of the desired DNA into the plant host cell under conditions where replication and normal expression will occur. This will also usually include transfer of the marker, so that cells containing the desired DNA may be readily selected.

The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the production of vegetable oils. These plants include,

but are not limited to rapeseed, peanut, sunflower, *C. tinctorius*, cotton, *Cuphea*, soybean, and corn or palm.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

In addition, synthase I or II produced in accordance with the subject invention can be used in preparing antibodies for assays for detecting plant synthases from other sources. The plant synthases can also be used in conjunction with chloroplast lysates to enhance the production and/or modify the composition of the fatty acids prepared *in vitro*. The plant synthase can also be used for studying the mechanism of fatty acid formation in plants and bacteria.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Materials

Commercially available biological chemicals and chromatographic materials, including Reactive Green 19 agarose, Ponceau S, ammonium carbonate, sodium borohydride ($NaBH_4$), malonyl CoA, free fatty acids, β-mercaptoethanol, and protease inhibitors amino caproic acid, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride are from Sigma (St. Louis, MO). CNBr-activated Sepharose 4B is purchased from Pharmacia (Piscataway, NJ). Proteolytic enzymes, including trypsin and endoproteinase gluC, are sequencing

grade enzymes obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Polyvinylidenefluoride (PVDF) membranes are Immobilon-P (Millipore, Bedford, MA). HPLC grade acetonitrile, methanol, chloroform and water are obtained from Burdick and Jackson (Muskegon, MI). Organic solvents including glacial acetic acid and methanol are from J.T. Baker (Phillipsburg, NJ). Tetrahydrofuran, dimethylphenylthiourea (DMPTU), and HPLC grade trifluoroacetic acid (TFA) are from Applied Biosystems (Foster City, CA); all chemicals utilized in the Applied Biosystems 477A Sequencer (see below) are also from Applied Biosystems (ABI). Radiochemicals, including ^{14}C-malonyl-Coenzyme A, [9,10(n)-3H] oleic acid (10mCi/mmol), and decanoic acid are from New England Nuclear (NEN (Dupont), Boston, MA.). [3H]-iodoacetic acid is from Amersham, (Arlington Heights, IL).

Example 1. FAS Analysis

In this example, the effects of additions of partially purified β-ketoacyl-ACP synthase and/or Δ-9 desaturase to FAS systems in cell-free extracts are described.

A. Synthase Experiment

1. Purification of *S. oleracea* Leaf Synthase II.

1.1 Assay. Synthase II activity is detected radiometrically as described in Example 2.

1.2 Purification. β-ketoacyl-ACP synthase II is partially purified from *S. oleracea* leaf according to the protocol of Shimakata and Stumpf (*PNAS* (1982) 79:5808-5812), with the following changes.

The Sephacryl S300 (Pharmacia) column is 2.5 cm x 100 cm and the flow rate is 18 ml/hour. Synthase I and II activities co-elute in a single peak.

The Affi-gel blue-agarose column (Bio-Rad, Richmond, CA) is 1.5 cm x 14 cm and the flow rate is 10 ml/hour. The synthase II activity requires 300 mM phosphate for elution.

The Whatman (Clifton, NJ) P11 cellulose phosphate column, 1.5 cm x 14 cm, is run at 9 ml/hour. As phosphate

at 50 mM potassium phosphate as was reported by Shimakata and Stumpf (*supra*), the phosphate salt concentration is reduced to 10 mM both in the column buffer and in the applied sample. The synthase I activity does not adsorb to the column even at 10 mM potassium phosphate and is recovered in the flow-through. The synthase II activity is eluted step-wise at 100 mM phosphate.

1.3 Specific Activity. One unit of activity is defined as the amount of protein required for the formation of 1 µmol of β-ketoacyl-ACP condensation product per minute. This is measured as incorporation of 1 µmole [2-^{14}C]malonate, supplied as [2-^{14}C]-malonyl-COA, into β-ketoacyl-ACP per minute at 37°C under assay conditions. The recovery of synthase II activity was 164 mU from 30 g *S. oleracea* leaves, with a specific activity of 31.6 mU/mg protein. The recovery of activity was 35%. Protein is measured by the Bradford method (*Analy. Biochem.* (1976) *72*:248-254).

2. Substrate Specificity.

The partially purified *S. oleracea* leaf synthase II activity is twelve times more active with the palmitoyl-ACP substrate than with decanoyl-ACP. In order to show that the small amount of decanoyl-ACP activity observed is from synthase II, the activity is tested for sensitivity to cerulenin, an irreversible inhibitor of synthase I activity. Since the synthase I activity does not adsorb to the cellulose phosphate, the activity in the flow-through from that column is compared to the synthase II activity peak from cellulose phosphate. Each enzyme source is incubated with 5 µM cerulenin for 15 minutes at room temperature before being assayed with each of the acyl-ACP substrates. After incubation with cerulenin, the decanoyl-ACP activity is reduced 99% in the cellulose phosphate flow-through fraction and only 69% in the synthase II fraction. The palmitoyl-ACP activity in the synthase II fraction is not inhibited by the cerulenin treatment.

3. Preparation of Cell-free Extracts.

Brassica napus is grown in the greenhouse under a 16-hour photoperiod with temperatures ranging from 76°F(days) to 60°F (nights). The seedpods are collected at 24-28 days after flowering. The seeds are removed from the pods, frozen in liquid nitrogen, and ground with a mortar and pestle. The powder is triturated with two volumes (2 ml/g fresh weight) of buffer containing 50 mM potassium phosphate, 2 mM dithiothreitol, pH 7.5. The sample is mixed by vortex for one minute and clarified by centrifugation at 10,000 x g for 15 minutes. Soluble protein is measured by the Bradford method (*supra.*). A clarified homogenate is made from *S. oleracea* leaves according to the protocol of Shimakata and Stumpf (1982, *supra.*) through the step involving centrifugation at 10,000 x g.

The extracts are assayed for endogenous β-ketoacyl-ACP synthase II activity as described in Example 2. The synthase II activity in the *S. oleracea* leaf extract was 228 μU/mg protein. The synthase II activity in the *Brassica* developing seed extract was 1200 μU/mg protein.

4. Fatty Acid Synthetase Reaction.

The FAS reaction includes in a 250 μl volume 100 mM HEPES-NaOH buffer, pH 8.0, 2 mM dithiothreitol, 400 μM each NADH and NADPH, 10.4 μM ACP, 12 μM acetyl coenzyme A, 5 μM [2-14C]malonyl coenzyme A (NEN, specific activity 55 Ci/mol), 300 μg protein from *B. napus* seed or *S. oleracea* leaf cell-free extracts, and varying amounts of partially purified synthase II.

The partially purified synthase II from *S. oleracea* leaf is added to the *S. oleracea* leaf FAS reaction mix at 1-, 2-, and 10-fold excess, and to the *Brassica* seed FAS reaction mix at 0.2-, 0.4-, and 2-fold excess. The reactions are incubated for ten minutes at 33°C and are stopped by the addition of 1 ml of hexane-isopropanol (3:2, v:v) and 667 μl sodium sulfate from a 6.67% (w/v) solution. The phases are mixed by vortex for 30 seconds and separated

by centrifugation at 4,500 x g for one minute. The hexane-isopropanol layer is removed by aspiration, and the sample is re-extracted. The hexane-isopropanol extracts are combined and an aliquot is counted in Aquasol (NEN) in a Beckman (Fullerton, CA) LS7800 liquid scintillation counter.

5. Detection.

The hexane-isopropanol is evaporated from the extracts under a stream of nitrogen gas and the lipids are saponified in 200 µl methanol with 100 µM potassium hydroxide for thirty minutes at 80°C. The saponified lipids are cooled to room temperature and titrated to pH 8.5 with 1 N hydrochloric acid in methanol to a phenolphthalein endpoint. The methanol is evaporated under a stream of nitrogen gas. Phenacyl esters of the fatty acids in each sample are prepared by incubation with 100 µl *p*-Bromophenacyl-8 (Pierce, Rockford, IL) and 300 µl acetonitrile at 80°C for 30 minutes. The sample is cooled to room temperature, 40 µl HPLC-grade water is added, and the solution is clarified by centrifugation at 4,500 x g for five minutes. The supernatant fluid is injected on to an Ultrasphere ODS reverse-phase HPLC column (5 micron particle size, 4.6 mm x 25 cm; Beckman, Fullerton, CA) equilibrated in 80% acetonitrile in water. The fatty acid phenacyl esters are eluted at one ml per minute in 80% acetonitrile in water for 10 min., followed by a one minute gradient of 80-100% acetonitrile in water, and finally, by 100% acetonitrile for 19 min.. Elution of the esters is monitored by UV absorbance and by radioactivity.

In both the *S. oleracea* leaf and the *Brassica* developing seed extracts, with an increase in the amount of synthase II added to the FAS system, the percent of radioactivity in palmitic acid decreases with a corresponding increase in the radioactivity detectable in stearic acid. In the table below, the radioactivities in the palmitate and the stearate are expressed as the percent of the total radioactivity recovered in fatty acids.

TABLE III

Effect of *S. oleracea* Leaf ß-ketoacyl-ACP Synthase II on the *de novo* Synthesis of Fatty Acids in Cell-free Extracts of *S. oleracea* Leaf and of Developing Seeds of *Brassica napus*

Synthase II Added (fold excess over endogenous)	C16:0 %	C18:0 %
S. oleracea Leaf		
0	58.4 ± 2.7	41.6 ± 2.7
1X	36.1 ± 0.0	57.9 ± 6.1
2X	34.3 ± 1.8	64.0 ± 0.2
10X	10.2 ± 1.0	73.0 ± 1.5
Brassica seed		
0	48.0 ± 5.0	48.9 ± 1.9
0.2X	37.5 ± 1.1	59.0 ± 2.6
0.4X	37.3 ± 3.4	59.8 ± 0.5
2X	29.9 ± 1.3	68.2 ± 0.7

B. Synthase II and Desaturase Experiment

1. Purification of *S. oleracea* Leaf Synthase II.

β-ketoacyl-ACP synthase II is partially purified from *S. oleracea* leaves as described above. The synthase II activity from the cellulose phosphate column is concentrated in a Centriprep 30 (Amicon Corporation, Danvers, MA). During storage at -70°C, the enzyme loses activity. When this experiment was conducted, the preparation had a specific activity of 10.4 mU/mg protein, and the concentration was 42 mU/ml. Protein was measured by the micro-method of Bradford (*supra.*) with BioRad Protein Assay Dye Reagent. One unit of activity is defined as the amount of protein required for the incorporation of 1 µmole [2-^{14}C]malonate into the reduced product per minute at 37°C.

2. Purification of *C. tinctorius* Seed Δ-9 Desaturase.

2.1. Substrate. In each of the following steps, the presence of the enzyme is detected radiometrically by measuring enzyme-catalyzed release of tritium from [9,10(n)-^{3}H]stearoyl-ACP, which is prepared and purified from synthesized [9,10(n)-^{3}H]stearic acid (synthesis described below) by the enzymatic synthesis procedure of Rock, Garwin, and Cronan (*Methods in Enzymol.* (1981) *72*:397-403).

[9,10(n)-^{3}H]stearic acid is synthesized by reduction of [9,10(n)-^{3}H]oleic acid with hydrazine hydrate essentially as described by Johnson and Gurr (*Lipids* (1971) *6*:78-84). [9,10(n)-^{3}H]oleic acid (2 mCi), supplemented with 5.58 mg unlabeled oleic acid to give a final specific radioactivity of 100 mCi/mmol, is dissolved in 2 ml of acetonitrile, acidified with 40 µl of glacial acetic acid, and heated to 55°C. Reduction is initiated with 100 µl of 60% (w/w) hydrazine hydrate; oxygen is bubbled through the mixture continuously. After each hour acetonitrile is added to bring the volume back to 2 ml and an additional 100 µl of hydrazine hydrate is added. At the end of 5 hr. the reaction is stopped by addition of 3 ml of 2M HCl. The reaction products are extracted with three 3-ml aliquots of petroleum ether and the combined ether extracts are washed with water, dried over sodium sulfate and evaporated to dryness. The dried reaction products are redissolved in 1.0 ml acetonitrile and stored at -20°C. The distribution of fatty acid products in a 15 µl aliquot is determined by preparation of phenacyl esters, which are then analyzed by thin layer chromatography on C-18 reverse phase plates developed with methanol:water::95:5 (v/v). Usually reduction to [9,10(n)-^{3}H]stearic acid is greater than 90%, a small amount of unreacted oleic acid may remain. The analysis is used to establish fraction of the total radioactivity that is present as stearate, and thereby to determine the exact substrate concentration in the enzyme assay.

2.2 Assay. The assay is performed by mixing 150 µl water, 5 ml dithiothreitol (100 mM, freshly prepared in water), 10 µl bovine serum albumin (10 mg/ml in water), 15 µl NADPH (25 mM, freshly prepared in 0.1 M Tricine-HCl, pH 8.2), 25 µl *S. oleracea* ferredoxin (2 mg/ml Sigma Type III in water), 3 µl NADPH:ferredoxin oxidoreductase (2.5 units/ml from Sigma), and 1 µl bovine liver catalase (800,000 units/ml from Sigma); after 10 min at room temperature, this mixture is added to a 13x100 mm screw-cap test tube containing 250 µl sodium 1,4-piperazinediethanesulfonate (0.1 M, pH 6.0). Finally, 10 µl of the sample to be assayed is added and the reaction is started by adding 30 µl of the substrate, [9,10(n)-^{3}H]stearoyl-ACP (100 µCi/µmol, 10 µM in 0.1 M sodium 1,4-piperazinediethanesulfonate, pH 5.8). After sealing with a cap, the reaction is allowed to proceed for 10 min. with shaking at 23°C. The reaction is terminated by addition of 1.2 ml of 5.8% tricholoracetic acid and the resulting precipitated acyl-ACPs are removed by centrifugation. The tritium released into the aqueous supernatant fluid by the desaturase reaction is measured by liquid scintillation spectrometry. One unit of activity is defined as the amount of enzyme required to convert one µmol of stearoyl-ACP to oleoyl-ACP, or to release 4 µmol of ^{3}H per minute.

2.3 Source Tissue. Developing *Carthamus tinctorius* (safflower) seeds from greenhouse grown plants are harvested between 16 and 18 days after flowering, frozen in liquid nitrogen and stored at -70°C until extracted.

2.4 Acetone Powder Extract. Approximately 50 g of frozen *C. tinctorius* seeds are ground in liquid nitrogen and sieved to remove large seed coat pieces to provide a fine embryo powder. The powder is washed with acetone on a Buchner funnel until all yellow color is absent from the filtrate. The powder is then air dried and further processed as described below, or may be stored frozen for at least a year at -70°C without loss of enzyme activity. The dried acetone powder is weighed and triturated with ten times its weight of 20 mM potassium phosphate, pH 6.8; the

mixture is then centrifuged at 12,000 x g for 20 minutes and decanted through a layer of Miracloth (Calbiochem, La Jolla, CA).

2.5 Ion Exchange Chromatography. The acetone powder extract is then applied to a DEAE-cellulose column (Whatman DE-52) (1.5 x 12 cm) equilibrated with 20 mM potassium phosphate, pH 6.8. The pass-through and a wash with one column-volume (20 ml) of buffer are pooled.

2.6 Affinity Chromatography. An affinity matrix for purification of the desaturase is prepared by reacting highly purified *E. coli* ACP, with CNBr-activated Sepharose 4B (Sigma). ACP (120 mg) is reduced by treatment with 1 mM dithiothreitol for 30 min on ice, and then desalted on Sephadex G-10 (Pharmacia) equilibrated with 0.1 M sodium bicarbonate, pH 6.0. The treated ACP (20 ml, 6 mg/ml) is then mixed with 20 ml of CNBr-activated Sepharose 4B swollen in 0.1 M sodium bicarbonate, pH 7.0, and the mixture is allowed to stand at 4°C for one day. The gel suspension is then centrifuged, washed once with 0.1 M sodium bicarbonate, pH 7.0, and then treated with 40 ml 0.1 M glycine, pH 8.0, for 4 hours at room temperature to block unreacted sites. The gel is then washed for five cycles with alternating 50 ml volumes of 0.5 M NaCl in 0.1 M sodium acetate, pH 4.0, and 0.5 M NaCl in 0.1 M sodium bicarbonate, pH 6.5, to remove non-covalently bound ligand. The gel is loaded into a column (1.5 x 11.2 cm) and equilibrated in 20 mM potassium phosphate, pH 6.8.

The combined fractions from the DE-52 column are applied to the column, which is subsequently washed with one column volume (20 ml) of the equilibration buffer, and then with 2.5 column volumes (50 ml) of 300 mM potassium phosphate, pH 6.8. Fractions are assayed for protein using the BCA Protein Assay Reagent (Pierce, Rockford, IL) to make sure that all extraneous protein has been eluted. Active Δ-9 desaturase is eluted from the column with 600 mM potassium phosphate, pH 6.8. Active fractions are pooled and concentrated using an Amicon 8400 stirred pressure cell. The protein preparation had a specific activity of

0.131 mU/mg protein, and the concentration was 3.15 mU/ml. The desaturase activity is extremely unstable making it necessary to proceed with the experiment immediately following the final step in the desaturase purification.

3. Preparation of cell-free extracts.

Cell-free extracts are prepared from *B. napus* seeds as described in Example 1A. Soluble protein is measured by the micro-method of Bradford (*supra.*) with BioRad Protein Assay Dye Reagent. The *B. napus* extract is assayed for endogenous β-ketoacyl-ACP synthase II activity as described in Example 2, and for endogenous stearoyl desaturase activity as described above. In the *Brassica* developing seed extract, the synthase II activity was 1280 μU/mg protein; the desaturase activity was 131 μU/mg protein.

4. Fatty Acid Synthetase Reaction.

The FAS reaction includes in a 250 μl volume 100 mM HEPES-NaOH buffer, pH 8.0, 2 mM dithiothreitol, 400 μM each NADH and NADPH, 10.4 μM ACP, 12 μM acetyl coenzyme A, 5 μM [2-14C]malonyl coenzyme A (NEN, specific activity 55 Ci/mol), 150 μg *Brassica* developing seed protein from cell-free extracts, and *S. oleracea* leaf synthase II and/or *C. tinctorius* seed desaturase.

The partially purified β-ketoacyl-ACP synthase II from *S. oleracea* leaf is added to the *Brassica* seed FAS reaction mix at 2.8- and 12.6-fold excess. The partially purified stearoyl desaturase is added at 2.1- and 9.3-fold excess. The reactions with no exogenous synthase or desaturase contain instead the equivalent volume of the synthase or desaturase buffer. The reactions are incubated for ten minutes at 33°C and stopped by the addition of 1 ml of hexane-isopropanol (3:2, v:v) and 667 μl sodium sulfate from a 6.67% (w/v) solution. The phases are mixed by vortex for 30 seconds and then separated by centrifugation at 4500 x g for one minute. The hexane-isopropanol layer is removed by aspiration, and the sample is re-extracted. The hexane-isopropanol extracts are combined and an aliquot

is counted in Aquasol (NEN) in a Beckman LS7800 liquid scintillation counter.

5. Detection.

The hexane-isopropanol is evaporated from the extract under a stream of nitrogen gas and the lipids are saponified in 200 µl methanol with 100 µM potassium hydroxide for thirty minutes at 80°C. The saponified lipids are cooled to room temperature and titrated to pH 8.5 with 1 N hydrochloric acid in methanol, to a phenolphthalein endpoint. The methanol is evaporated under a stream of nitrogen gas. Phenacyl esters of the fatty acids in each sample are prepared by incubation with 100 µl *p*-Bromophenacyl-8 (Pierce, Rockford IL) and 100 µl acetonitrile at 80°C for 30 minutes. The sample is cooled to room temperature, and the solution clarified by centrifugation at 4500 x g for five minutes. The supernatant fluid is injected to an Ultrasphere ODS reverse-phase HPLC column (5 micron particle size, 4.6 mm x 25 cm) (Beckman, Fullerton, CA) equilibrated in 82% acetonitrile in water. The fatty acid phenacyl esters are eluted at two ml per minute in 82% acetonitrile in water for 100 minutes, followed by 25 minutes in 100% acetonitrile. Elution of the exters in monitored by UV absorbance and by radioactivity.

As shown in the tables below, with increasing amounts of *S. oleracea* leaf synthase II added to the *Brassica* FAS system, the amount of radioactivity in palmitic acid decreases with a corresponding increase in the radioactivity in stearic acid. With increasing amounts of *C. tinctorius* seed desaturase, the amount of radioactivity decreases in saturated fatty acids measured (palmitic acid and stearic acid) with a corresponding increase in the amount of radioactivity incorporated into oleic acid. When the largest volume of desaturase is added to the *Brassica* FAS, the total incorporation of ^{14}C-malonyl-Coenzyme A into fatty acid decreases. This effect is probably due to components of the desaturase buffer interfering with the in

vitro FAS system and therefore limiting the amount of exogenous desaturase that can be added.

TABLE IV

Effects of *S. oleracea* Leaf β-ketoacyl-ACP Synthase II and *C. tinctorius* Seed Stearoyl Desaturase on the Incorporation of ^{14}C Malonyl CoEnzyme A into Lipids by a Cell-free Extract of Developing Seeds of *Brassica napus*

Synthase II Added (fold excess over endogenous)	Desaturase Added (fold excess over endogenous)	Incorporation cpm
0	0	20,726
0	2.1	20,971
0	9.3	10,253
2.8	0	20,787
2.8	2.1	21,329
2.8	9.3	9,629
12.6	0	19,650
12.6	2.1	19,409
12.6	9.3	10,736

In the table below, the radioactivities in the individual fatty acids are expressed as the percent of the total radioactivity recovered in fatty acids. The values are averages of duplicate syntheses within one experiment. Replicates were not available for the last two experimental conditions listed in the table.

TABLE V

Effects of *S. oleracea* Leaf β-ketoacyl-ACP Synthase II and *C. tinctorius* Seed Δ-9 Desaturase on the Distribution of Fatty Acids Synthesized *de novo* in a Cell-free Extract of Developing Seeds of *Brassica napus*

Synt.II Added (fold excess over endogenous)	Desat. Added	C16:0 %	C18:0 %	C18:1 %
0	0	12.2 ± 0.4	47.3 ± 1.2	36.0 ± 0.8
0	2.1	11.2 ± 0.6	40.3 ± 1.0	45.4 ± 0.4
0	9.3	17.4 ± 1.3	25.6 ± 3.5	55.6 ± 3.6
2.8	0	6.4 ± 0.3	65.6 ± 2.0	18.7 ± 1.3
2.8	2.1	5.9 ± 0.1	64.7 ± 0.1	24.8 ± 0.3
2.8	9.3	11.5 ± 0.3	50.2 ± 1.1	30.7 ± 2.0
12.6	0	7.1 ± 0.2	75.7 ± 0.1	6.6 ± 0.7
12.6	2.1	6.2	77.5	10.9
12.6	9.3	9.6	64.8	12.4

Example 2. Assay for Synthase Activity

In this example, the assay used to detect synthase activity is described. The presence of the synthase activity is detected radiometrically by modification of the method of Garwin *et al.* (*J. Biol. Chem.* (1980) *255*:11949-11956), by measuring synthase-catalyzed condensation of [2-^{14}C]malonyl-ACP with either decanoyl-ACP (C10-ACP) or hexadecanoyl-ACP (C16-ACP), which produces β-ketododecanoyl-ACP (C12-ACP) or β-ketooctadecanoyl-ACP (C18-ACP), respectively. Products are reduced to their 1,3,-diol forms for extraction into toluene prior to determining incorporation by scintillation counting.

A. Assay

The synthase assay contains 0.2 M potassium phosphate (pH 6.8), 1.25 mM EDTA, 3.1 mM β-mercaptoethanol, 10 μ Units malonyl CoA-ACP transacylase (MTA) (purification from *E. coli* described below), 50 μM ACP (purification from *E.*

coli described below), 100 μM [2-^{14}C]malonyl-CoA (20 Ci/mol), 60 μM C16-ACP or C10-ACP, 5% glycerol and enzyme in a total reaction volume of 20 μl.

For maximal activity the MTA and ACP are reduced prior to the enzyme assay by incubation of 1 μl of 1 mM ACP and 0.08 ml of MTA (at 129 μU/ml) in 0.4 μl of 50 mM EDTA, 0.6 μl of 20 mM β-mercaptoethanol, 2 μl of 1 M potassium phosphate, and 1.12 μl of H_2O for 15 minutes at 37°C. This solution is then added to a 7.3 μl solution containing 2 μl of 1 M potassium phosphate, 1.3 μl of 1 mM malonyl-CoA and 4 μl of [2-^{14}C]malonyl-CoA (47.8 Ci/mol), and the mixed solution is incubated for 2-5 minutes at room temperature to allow MTA to reach equilibrium. Acyl-ACP, either C16-ACP or C10-ACP, in a total volume of 2.5 μl is added to the above solution and the samples are placed at 37°C. The reaction is started by addition of 5 μl of enzyme which is in potassium phosphate buffer (pH 7.5), 20% glycerol (V/V), 1mM EDTA, and 10 mM β-mercaptoethanol.

The reaction is stopped after 15 minutes by addition of 400 μl of reducing agent containing 0.1 M potassium phosphate, 0.4 M potassium chloride, 30% tetrahydrofuran, and 5 mg/ml $NaBH_4$, with the $NaBH_4$ being added just prior to use. Tubes are vortexed to mix thoroughly after addition of reducing agent and then are incubated for at least 30 minutes (and for up to 3 hours) at 37°C. Toluene, 0.4 ml, is added and samples are again vortexed to mix. Samples are centrifuged for 10 seconds in a microcentrifuge to separate phases. 300 μl of the toluene layer (upper phase) is added to 5 ml Aquasol (NEN Research Products (Dupont) Boston, MA) and incorporation of [2-^{14}C]malonyl CoA is determined by scintillation counting.

B. Preparation of Assay Components

1. Purification of Acyl Carrier Protein from *E. coli*.

1.1 Assay for ACP. The assay for ACP includes in a volume of 40 μl, 100 mM Tris-HCl, pH8.0, 1% Triton X-100 (w/v) (Boehringer Mannheim Biochemicals, Indianopolis, IN), 2 mM dithiothreitol, 5mM adenosine triphosphate, 20 mM $MgCl_2$, 300 mM LiCl, 33.5 μM [1-^{14}C]palmitic acid (NEN

Research Products (Dupont), Boston, MA, specific activity 56 Ci/mol), 32 mU of acyl-ACP synthetase (purified as described below), and 5 µl of ACP source to be assayed. Each assay tube is centrifuged briefly to collect all the liquid into the bottom of the tube, then for three hours at 37°C. From each assay tube 30 µl of the 40 µl volume is dropped onto a 1 cm^2 piece of filter paper, numbered in pencil and suspended on a straight pin. The filters are allowed to air dry for at least 45 minutes, then are washed two times in chloroform:methanol:acetic acid (3:6:1, v:v:v) at the ratio of 15 ml/filter, in a beaker with stirring. The filters are placed into scintillation vials and counted in 5ml Aquasol in a Beckman LS3801 liquid scintillation counter (Beckman, Fullerton, CA). A one-to-one stoichiometry is assumed between ACP and the 1-^{14}C-palmitate with the product, ^{14}C-palmitoyl-ACP, bound to the filter, and the free fatty acid washed into the organic solvent.

1.2 Extraction and Purification of ACP. The acyl carrier protein (ACP) is purified from *E. coli* strain K-12 by a modification of the method of Rock and Cronan, (*Methods in Enzymol.* (1981) 71:341-351). The *E. coli* is obtainable from Grain Processing Corporation (Muscatine, IA) as frozen late-logarithmic phase cells. One kilogram of frozen packed cells of *E. coli* K12 is thawed overnight at 4°C. At room temperature, the cells are combined with 1 L deionized water, 500 mg lysozyme (from chicken egg white, Sigma), and 200 ml lysis buffer containing 1M Tris-HCl, 1M glycine, 250 mM sodium EDTA, pH 8.0. This is stirred at room temperature by a Wheaton overhead stirrer. After two hours, 5 g of Triton X-100 is added and stirring is continued for another hour. The suspension is further homogenized by blending in a 6L Waring Model CB6 Commercial Blender for 60 seconds. Stirring of the homogenate is resumed and 2 L of isopropanol are added from a separatory funnel in a thin stream over twenty minutes to ensure immediate mixing.

Immediately after the addition of the isopropanol, the homogenate is clarified by centrifugation at 10,000 x g for 30 minutes at room temperature. The pH of the supernatant fluid is adjusted to 6.5 with glacial acetic acid and this is combined with 440 ml of a 50% slurry of Whatman DE52 (Whatman, Clifton, NJ) in 10 mM bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane hydrochloride (Bis-Tris-Hcl) (Sigma, St. Louis, MO), pH 6.5. It is important to sediment the precipitate and combine the supernatant with the DE52 as quickly as possible to prevent clogging of the filters and the column in subsequent steps. The DE52 slurry is allowed to stir overnight or for at least one hour at room temperature, and is then filtered on a sintered glass funnel. The DE52 in the filter funnel is washed five times with 200 ml 10 mM Bis-Tris-Hcl, 2 mM DTT, 0.1% (w/v) TX-100, and four times with 200 ml 10 mM Bis-Tris-Hcl, 2 mM DTT, 250 mM LiCl. It is resuspended in 200 ml of the last wash buffer and poured into a 4.8 cm x 40 cm column. The flow-through is collected in bulk. The ACP is eluted at 2 ml/min with 10 mM Bis-Tris-Hcl, 2 mM DTT, 600 mM LiCl; 6 ml fractions are collected.

After it was determined by the radiometric assay that the ACP fractions also contain a yellow contaminant, the ACP fractions were selected for pooling by their yellow color. The fractions are pooled into a centrifuge bottle and with stirring on ice, the ACP is precipitated by the dropwise addition of 50% ice cold trichloroacetic acid (TCA) to a final concentration of 5% TCA. The protein is quickly sedimented by centrifugation at 10,000 x g for 30 minutes at 4°C.

The pellet is suspended in 10 ml deionized water, and solubilized by the addition of 500 µl aliquots of 1 M Tris base, with homogenization in a hand-held ground glass homogenizer after each addition. At pH 7.0, the solution is clear. The volume is brought to 50 ml with deionized water, and contaminating protein is precipitated by the addition of 26.15 g (80% saturation) solid ammonium sulfate with stirring on ice over 45 minutes. Stirring is

continued for one hour on ice, and the protein is sedimented by centrifugation at 23,700 x g for 45 minutes. The ACP is concentrated by precipitation with trichloroacetic acid and resolubilized with Tris base as described above, keeping the final volume to about 5ml or less. The solution is clarified by centrifugation at 20,000 x g for 20 minutes at 4°C. A typical yield of ACP is 80-100 mg from 1 kg packed cells of *E. coli* K12.

2. Purification of Malonyl Coenzyme A:Acyl Carrier Protein Transacylase (MTA) from *E. coli*.

2.1 MTA Assay. The MTA activity is measured at room temperature by modification of the the assay of Alberts *et al.* (*Methods in Enzymol.* (1969) *14*:53-56). The reaction mix of 100 µl includes 100 mM potassium phosphate buffer, pH 6.5, 100 µM ACP, 100 µM malonyl coenzymeA (Sigma), 10 nCi [2-^{14}C]malonyl coenzyme A (specific activity 43.1 Ci/mol, NEN), 2 mM β-mercaptoethanol and enzyme in 10 µl. The ACP is incubated with an equal volume of 20 mM β-mercaptoethanol for 15 minutes before being added to the rest of the reaction mix excluding the enzyme. The reaction is started by the addition of enzyme, incubated for 4 minutes at 23°C, and stopped by the addition of 400 µl ice cold 5% perchloric (v/v) acid. The reaction is held on ice for at least 20 minutes. The precipitate is collected on a Millipore HA, 0.45 micron filter on a Millipore filtration manifold (Millipore, Bedford, MA). The precipitate from each assay tube is washed on the filter three times with 5 ml ice cold 5% (v/v) perchloric acid. The filters are dropped into 5 ml Aquasol (NEN) without neutralization or drying, and are counted in a Beckman LS3801 Liquid Scintillation Counter (Beckman, Fullerton, CA). One unit of MTA activity is defined as the conversion of 1 µmole of acid-soluble malonate to acid-precipitable malonate per minute. Protein determinations are by the micromethod of Bradford (*Analy. Biochem.* (1976) *72*:248-254), using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Richmond, CA).

2.2 Extraction and Purification of MTA. The MTA is purified from E. coli by a modification of the method of Alberts et al. (*Methods in Enzymol.* (1969) *14*:53-56). All steps of the purification are carried out at 4°C. Fractions are routinely stored overnight at 4°C between steps of the protocol. Frozen packed cells (20 g) of *E. coli* K12 available from Grain Processing Corporation (Muscatine, IA) are thawed overnight, then suspended in 20 ml Buffer A which contains 10 mM Tris-HCl, 1 mM sodium EDTA and 10 mM β-mercaptoethanol at pH 7.5. The cells are ruptured by two passes through a French Pressure Cell (American Instrument Company, Silver Spring, MD) at 16,000 psi. The broken cells are diluted with 40 ml of Buffer A and the particulates are sedimented by centrifugation at 16,000 x g for 30 minutes. The supernatant is combined with streptomycin sulfate (Sigma) from a 20% solution in Buffer A (w/v) for a final concentration of 60 mg streptomycin sulfate per milliliter. The precipitate is sedimented immediately by centrifugation at 17,500 x g for 15 minutes. The supernatant fluid is diluted with three volumes of Buffer B, which contains 10 mM potassium phosphate, 1 mM sodium EDTA and 10 mM β-mercaptoethanol at pH 7.0, and applied at 360 ml/h to a 4.8 cm x 11 cm column of DEAE Fast-Flow (Pharmacia, Piscataway, NJ) equilibrated in Buffer B. The column is washed with two bed volumes of Buffer B containing 75 mM LiCl. The MTA activity is eluted with a two-bed volume gradient of Buffer B containing 75-250 mM LiCl.

The fractions containing peak MTA activity, at about 240 mM LiCl, are pooled and non-MTA protein is precipitated by the addition of solid ammonium sulfate (29.5 g/100ml.) The suspension is stirred for 30 minutes, then clarified by centrifugation at 16,000 x g for 30 minutes. The MTA activity is precipitated by the addition of solid ammonium sulfate to the supernatant fluid (19.7 g/100 ml.) which is stirred and sedimented by centrifugation as above. The pellet is suspended with 10 ml Buffer C, containing 20 mM potassium phosphate, 1 mM sodium EDTA and 10 mM β-

mercaptoethanol at pH 7.0 and applied to a 2.5 cm x 40 cm column of Sephadex G-100 (Pharmacia Inc., Piscataway, NJ) equilibrated in Buffer C. The protein is eluted at 60 ml/h. The fractions containing peak MTA activity are pooled and applied at 60 ml/h to a 2.5 cm x 4.7 cm column of DEAE Fast-Flow in Buffer D, which is Buffer C containing 150 mM LiCl. The column is washed with three bed volumes of Buffer D, then a 4-bed volume gradient of Buffer C containing 150-300 mM LiCl is applied. The MTA activity elutes in the 150 mM LiCl wash rather than in the LiCl gradient as was described by Alberts *et al.* (supra) The fractions containing peak MTA activity are pooled and the protein is precipitated by the addition of ammonium sulfate (61.1 g/100 ml.) The suspension is stirred for one hour and the precipitated MTA activity is collected by centrifugation at 16,000 x g for one hour. The pellet is dissolved in 0.5 ml Buffer C and dialysed overnight against one liter of Buffer C. The dialysate is stored as aliquots in polypropylene tubes at -70°C.

About 3.7 units of MTA activity are produced from 20 g of packed cells by this method. When stored at -70°C, as a concentrate of 2.5 U/ml, the MTA retains 100% of its activity for at least 18 months. For the β-ketoacyl-ACP synthase assay, it is diluted into "20 buffer" (described below in Example 3) containing 10 mM β-mercaptoethanol.

3. Purification of Acyl-Acyl Carrier Protein Synthetase.

Acyl-ACP synthetase is purified according to the method of Rock and Cronan, 1979, *J. Biol Chem*, Vol 254 No. 15:7116-7122, with the following changes. The heat treatment is eliminated. The fraction from the Blue-Sepharose column is applied at 240 ml/hour to a 2.4 cm x 40 cm column of BioGel P-6DG (Bio-Rad, Richmond, CA) equilibrated in 5 mM Tris-HCl, pH 8.0 with 2% (w/v) Triton X-100. Throughout the purification, protein levels are determined with the Micro BCA Protein Reagent from Pierce (Rockford, IL).

Although quite variable, the yield of acetyl-ACP synthetase is about 55 units from 125 g *E. coli* K12 packed

cells. One unit of activity is described as the amount of protein required to produce 1 nmol of C16:0-ACP from palmitate and ACP per minute. During storage at 4°C, a precipitate is formed that must be suspended before use.

4. Synthesis and Purification of Acyl-Acyl Carrier Protein.

The C10:0-ACP and C16:0-ACP substrates are synthesized enzymatically and are purified by the procedure of Rock *et al.* (*Methods in Enzymol.* (1981) *72*:397-403). The acyl-ACP substrates for the β-ketoacyl synthase I and II assays require no radiolabel, but sufficient ^{14}C or ^{3}H may be included to monitor the purification after the enzymatic synthesis. The yield of acyl-ACP is also monitored by the filter assay method described by Rock *et al.* (*supra*). The synthetic reaction includes, in a 10 ml volume, 4.8 μmol palmitic or decanoic free fatty acid, 800,000 cpm [9,10-^{3}H]palmitic acid (NEN Research Products (Dupont), Boston, MA, specific activity 28.5 Ci/mmol) or [1-^{14}C]decanoic acid (ICN Biomedicals, Inc, Costa Mesa, CA, specific activity 3.6 Ci/mol), 100 mM Tris-HCl pH 8.0, 5 mM adenosine 5'-triphosphate, 2 mM dithiothreitol, 2% Triton X-100 (Boehringer Mannheim Biochemicals, Indianopolis, IN), 400 mM LiCl, 10 mM $MgCl_2$, 15 mg ACP and 3 units acyl-ACP synthetase. The mix is incubated at 37°C for three hours and may be stored at room temperature for about 15 hours or at -20°C until purification.

The purification of the acyl-ACP from the synthetic mix is carried out at room temperature according to the procedure of Rock *et al.* (*supra*) with the following changes. The wash with 2-propanol is omitted as it was found that the free fatty acid does not bind to the DE-52 column. The volume of the wash of the DE-52 with equilibration buffer, however, is increased to ensure removal of the Triton X-100. Some lots of Octyl-Sepharose (Pharmacia Inc., Piscataway, NJ) do not allow adsorption of the acyl-ACP. A test column is therefore run with each new lot of the resin. In most cases, 50% 2-propanol is required for complete recovery of the acyl-ACP. The second

DE-52 column is omitted; instead, the acyl-ACP in 50% 2-propanol is brought to dryness by lyophilization or in a SpeedVac Concentrator (Savant Instruments Inc, Hicksville, NY), and is reconstituted with deionized water. The substrates are stored at -70°C.

Typical yields are 1.7 µmol of palmitoyl-ACP or 0.5 µmol of decanoyl-ACP.

Example 3. Purification of Synthase Proteins

In this example, purification of β-ketoacyl-ACP synthase from developing seeds of *Ricinus communis* is described. All steps of protein purification are done at 4°C or on ice. Until the ricin, a toxic seed protein, has been removed, all steps are done in a glove box or with appropriate precautions to prevent exposure to the toxin.

A. Extraction

1. Source tissue.

Ricinus communis is grown in the greenhouse under the following conditions.

: temperature range is 22-32°C

: lighting is set for a 16-hour day length (supplemental lighting is with high pressure sodium lamps)

: fertilizer is 60ppm nitrogen daily via watering

The developing seeds are harvested at 21 to 28 days after the flowers open. The endosperm is removed and frozen in liquid nitrogen, and is stored at -70°C for up to 18 months until used for purification of synthase activity.

2. Purification Buffers.

The buffers used in the purification of β-ketoacyl-ACP synthases are at pH 7.5, and contain 20% (v/v) glycerol, 1 mM sodium EDTA, 10 mM β-mercaptoethanol, and potassium phosphate at the millimolar concentration that is designated in the buffer name. For example, "20 buffer" contains 20 mM potassium phosphate. The exception is "zero buffer", which is lacking only in the potassium phosphate, and has a pH of approximately 5.

3. Extraction Procedure.

A 200 g batch of frozen tissue is homogenized in an Osterizer blender for 15 seconds at top speed in 400 ml of "40 buffer" which also contains the following protease inhibitors: 1 mM amino caproic acid, 1 μM leupeptin, 1 μM pepstatin, and 100 μM phenylmethylsulfonyl fluoride. Blending is limited to 15 seconds to prevent release of too much of the seed lipid, which if present would interfere with the subsequent ammonium sulfate fractionation. The crude homogenate is clarified by centrifugation at 16,000 x g for one hour. The supernatant fluid, Fraction A, contains the solubilized β-ketoacyl-ACP synthase activity.

B. Ammonium Sulfate Fractionation

Fraction A, is decanted through cheesecloth and Miracloth (CalBiochem, La Jolla, CA) and stirred with ammonium sulfate (40.4 g/100 ml) for one hour. The precipitated protein is sedimented by centrifugation at 16,000 x g for one hour. The supernatant fluid, Fraction B, containing the β-ketoacyl-ACP synthase activity, is stirred with ammonium sulfate (13.4 g/100 ml) for one hour and the protein sedimented by centrifugation at 16,000 x g for one hour. This ammonium sulfate pellet, Fraction D, is suspended in a minimal volume of "20 buffer" and stored at -70°C

C. Reactive Green 19 Agarose Chromatography

Fraction D is diluted with "20 buffer" to a final volume of 1650 ml. Fraction D is then combined with 200 ml (packed bed volume) Reactive Green 19-agarose (Sigma, St. Louis, MO) which has been pre-equilibrated in "20 buffer", and this solution is stirred by an overhead stirrer for two hours. The Green 19-agarose is filtered on a sintered glass funnel and washed with 200 ml of "50 buffer". The washed Green 19-agarose is suspended in an additional 175 ml of "50 buffer" and is poured into a 4.8 cm x 30 cm column. The agarose is packed at 200 ml/hr, until the last of the "50 buffer" reaches the top of the Green 19-agarose

bed. The synthase activity is then eluted with "250 buffer". The synthase activity elutes in the first 100 ml of "250 buffer". The fractions are stored at -20°C until the next step of purification.

D. ACP-Affinity Chromatography

An affinity matrix for purification of the synthase activities is prepared by reacting highly purified *E. coli* ACP, with CNBr-activated Sepharose 4B by modification of the procedure of McKeon and Stumpf (*J. Biol. Chem.* (1982) *257*:12141-12147) as described below.

1. Matrix Preparation.

E. coli ACP, 141 mg in a volume of 40 ml, is dialyzed against 3 volumes of 1 liter of 100 mM $NaHCO_3$, pH 6.0, for 24 hours in Spectrapor #7 dialysis tubing (molecular weight cutoff = 2000). One millimolar dithiothreitol is included in the second buffer change only, for a total of 3 hours. One hundred milliliters of 1 mM HCl is added to 6.0 g of CNBr-activated Sepharose in a 250 ml polypropylene centrifuge bottle. This is mixed at high speed for 15 minutes at room temperature on a "Rugged Rotator" (Kraft Apparatus, Inc., Mineola, NY) ferris-wheel type mixer. The resulting slurry is poured into a Kontes (Vineland, NJ) 2.5 cm x 20 cm column and is washed with 1.1 liter of 1 mM HCl at 250 ml/hr, at room temperature for the first two hours and then at 4°C. The CNBr-activated Sepharose is then washed in a 60 ml sintered glass funnel five times with 20 ml 100 mM $NaHCO_3$, pH 6.0, followed by 5 times with 20 ml 100 mM $NaHCO_3$, pH 7.0. The CNBr-activated Sepharose is then resuspended in 40 ml of 100 mM $NaHCO_3$, pH 7.0, and transferred to a clean 250 ml polypropylene centrifuge bottle. The dialyzed ACP is added to the CNBr-activated Sepharose slurry and the suspension is mixed for 24 hours at 4°C on the "Rugged Rotator" mixer to couple the ACP to the CNBr-activated Sepharose. The ACP-Sepharose is sedimented by centrifugation for 6 minutes at 100 x g (800 rpm in a Beckman GP tabletop swinging bucket centrifuge) at 4°C. The supernatant fluid is removed and saved for

assay. Fifty milliliters of 1 M ethanolamine at pH 8.0 is added to the ACP-Sepharose and mixed on the "Rugged Rotator" at 4°C for 16 hours to block unused binding sites.

2. Column Packing.

The ACP-Sepharose gel is poured into a 2.5 cm x 20 cm column and is washed with 100 ml of 100 mM $NaHCO_3$, pH 7.0 containing 500 mM NaCl, followed by another wash with 100 ml of 100 mM sodium acetate, pH 4.0 containing 500 mM NaCl. This washing with alternate buffers is repeated three times. The gel is then washed with 200 ml of the bicarbonate buffer, and finally with 200 ml of "20 buffer" plus 0.02% sodium azide. The gel is left in this buffer until further use. The unbound fractions were assayed for protein by the method of Bradford (Analy. Biochem. (1976) 72:248-254) and it was calculated that 134 mg of the ACP had bound to the 25 ml CNBr-activated Sepharose. Before application of protein sample, the ACP-Sepharose is packed into a 2.5 cm x 10 cm column and washed with 250 ml "20 buffer". Final volume of the packed bed is 25 ml.

3. Sample Preparation.

The fractions from the Green 19-agarose chromatography with synthase activity are combined in an Amicon 8400 stirred pressure cell apparatus with a PM30 membrane (Amicon, Danvers, MA). The emptied fraction tubes are rinsed with an equal volume of "zero buffer" and the rinse is added to the pooled fractions. Pressure is applied via nitrogen gas until the volume of the concentrate is 10% of the original solution volume. The pressure is then released and the stirring is continued for an additional 5 mins. The concentrate is diluted with "zero buffer" until the conductivity reaches that of "10 buffer", as measured with a Beckman conductivity meter.

4. Chromatography of Fractions from Green-19 Agarose.

The diluted sample containing synthase activity is loaded onto the ACP-Sepharose column at a flow rate of 100 ml per hour, and the column is washed with one bed volume

of "20 buffer". The flow-through and the "20 buffer" wash are collected in bulk, and 6 ml fractions are collected during elution. The protein is eluted at 25 ml per hour with five bed volumes of "100 buffer" followed by a ten bed volume gradient of "250 buffer". Following the gradient, the column is washed with additional "500 buffer" until a total of 72 fractions have been collected. The column is regenerated by washing with 10 bed volumes of "500 buffer", followed by 10 bed volumes of "20 buffer". When not in use, the column is stored in "20 buffer" containing 0.02% sodium azide as a preservative.

5. Assay of Fractions for Synthase Activity

The fractions were assayed for both C16-ACP and C10-ACP condensing activities. The major peak of C16-ACP condensing activity eluted in fractions 32-44. A portion of the C10-ACP activity eluted with the major C16-ACP condensing activity peak, but the bulk of the C10-ACP condensing activity eluted after the C16-ACP peak, sometimes as a broad shoulder to the initial peak, and sometimes as a separate peak.

E. SDS-PAGE Analysis and Separation of Peptides

The fractions of the ACP-Sepharose column are analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) by a variation of the method of Laemmli (Nature (1970) *227*:680-685). The resolving gel contains only 0.2% bis-acrylamide instead of the standard 0.267%. A Bio-Rad (Richmond, CA) Protean II mini-gel unit is used at 200V. The current is allowed to flow until the tracking dye reaches the end of the gel, and then for an additional 10 minutes. This allows better separation of the two major peptide bands. Fractions in the first peak of synthase activity, which contains the majority of the C16-ACP condensing activity, contain both a 46kD and a 50kD band with approximately equal silver-staining intensities. The bulk of the C10-ACP condensing activity, which elutes after the C16-ACP condensing activity, is associated with a more faintly staining 50 kD band. The synthase fractions are

pooled, concentrated and desalted and the proteins are separated in bulk by SDS-PAGE electrophoresis as described. From 200 g, fresh weight, of embryonic seed tissue, approximately 50 µg of each of the 46kD and 50kD proteins is recovered.

F. Purification Table

Protein recovery and synthese activity at each step of purification are presented in the tables below.

TABLE VI

Purification of *R. communis* ß-ketoacyl-ACP synthase I

	Total Protein (Bradford) mg	Protein Recovery %	Total Activity mol/min	Activity Recovery %	Specific Activity mol/min/mg	Purification -fold
crude extract	1059.4	100.00	13.3	100.00	.012	1.0
0-60% ammonium sulfate (supernatant)	386.6	36.49	33.1	249.12	.086	6.8
sat'd ammonium sulfate (pellet)	149.5	14.11	9.7	72.75	.065	5.2
Green-19 Agarose	18.3	1.73	.97	7.31	.053	4.2
ACP-Sepharose "Peak Two" (affinity chromatography)	0.01*	0.00	.16	1.22	16.2	1290.3

*rough estimate from Laemmli SDS-PAGE gel

TABLE VII

Purification of *R. communis* ß-ketoacyl ACP Synthase II

	Total Protein (Bradford) mg	Protein Recovery %	Total Activity mol/min	Activity Recovery %	Specific Activity mol/min/mg	Purification -fold
crude extract	1059.4	100.00	17.5	100.00	.016	1.0
0-60% ammonium sulfate (supernatant)	386.6	36.49	12.8	73.35	.033	2.0
sat'd ammonium sulfate (pellet)	149.5	14.11	10.0	57.26	.067	4.1
Green-19 Agarose	18.3	1.73	.93	5.29	.051	3.1
ACP-Sepharose (affinity chromatography)	0.09*	0.01	.15	0.87	1.70	102.9

*rough estimate from Laemmli SDS-PAGE gel

G. Electroblotting from SDS-PAGE

1. Gel Electrophoresis.

Material from the ACP-Sepharose column is applied to a 1.5 mm thick SDS reduced cross-linker mini-polyacrylamide gels prepared as described above in Example 3E. The resolving gel is poured the night before the gels are run; the stacking gel is poured the same day. Each ACP-Sepharose column pool contains 40-60 µg of protein. The protein is divided between 2 gels of 10 wells each, with each lane containing 2-3 µg of protein. The gels are run as described above in Example 3E. After electrophoresis, the gels are assembled into a Bio-Rad Mini Trans-Blot module (Bio-Rad, Richmond, CA) and the protein

is electroblotted to either nitrocellulose or polyvinylidenefluoride (PVDF) membranes.

2. Blotting to Nitrocellulose.

When protein is electroblotted to nitrocellulose, the blotting time is 1 hour and the buffer used is 25 mM Tris, 192 mM glycine in 20% methanol. Following electroblotting to nitrocellulose, membranes are stained in 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid for 2 minutes and destained in 2-3 changes of 0.1% (v/v) acetic acid, 2 minutes for each change. Following this, nitrocellulose membranes are stored wet in heat-sealed plastic bags at -20° C. Originally, nitrocellulose membranes were destained in 1% acetic acid and then rinsed with HPLC grade water prior to storage. The stained bands fade rapidly in neutral pH however, so the destain solution was changed to 0.1% acetic acid and membranes were also stored wetted in this solution. If time permits, blots are not frozen but used immediately for digestion to create peptides for determination of amino acid sequence as described below.

3. Blotting to PVDF.

When protein is electroblotted to PVDF, the blotting time is 30 minutes and the buffer used is 125 mM Tris/50 mM glycine in 10% (v/v) methanol. Following electroblotting to PVDF, membranes are stained in 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid for 5 minutes and destained in 2-3 changes of 50% (v/v) methanol/10% (v/v) acetic acid, 2 minutes for each change. Following this, PVDF membranes are allowed to air dry for 30 minutes and are then stored dry in heat-sealed plastic bags at -20° C. Protein blotted to PVDF is used directly to determine N-terminal sequence of the intact protein.

Example 4. Determination of Amino Acid Sequence

In this example, a method for the determination of the amino acid sequence of a plant β-ketoacyl-ACP synthase is described.

A. Digestion

Proteins blotted to nitrocellulose are subjected to digestion with proteases in order to obtain peptides for sequencing. The method used is that of Aebersold, *et al.* (*PNAS* (1987) *84*:6970). Bands of both the 46kD and 50kD proteins, and also an equal amount of blank nitrocellulose to be used as a control, are cut out of the nitrocellulose membrane, chopped into pieces 1 x 2 mm in size, and washed several times with HPLC grade water in order to remove the Ponceau S. The Ponceau S is not always removable if the blot has been frozen, but the presence of the stain apparently has no effect on the digest procedure. Following this wash, 1.0-1.2 ml of 0.5% polyvinylpyrrolidone (PVP-40, Aldrich, Milwaukee, WI) in 0.5% acetic acid is added to the membrane pieces and this mixture is incubated for 30 minutes at 37°C. The PVP-40 is needed to block sites on the nitrocellulose which would bind the protease and/or peptides released by the protease digestion. Following treatment with PVP-40, the membrane pieces are rinsed with several 1.0 ml volumes of HPLC grade water to remove excess PVP-40. The pieces are then suspended in either trypsin digest buffer, 100 mM sodium carbonate pH 8.2, or endoproteinase gluC buffer, 25 mM ammonium carbonate/1 mM EDTA, pH 7.8. Acetonitrile is added to the digest mixture to a concentration of 5% (v/v). Protease, trypson or endoproteinase glu C is diluted in digest buffer and added to the digest mixture in a ratio of 1:10 (w/w) protease to protein. Final volume of the digest mixture is 100 µl. Digests are incubated overnight. Trypsin digests are incubated at 37°C and endoproteinase gluC digests are incubated at room temperature.

B. Separation of Peptides

Following overnight incubation, digest reactions are stopped by the addition of 10µl 10% (v/v) trifluoroacetic acid (TFA). The digest mixture is removed from the nitrocellulose pieces, the nitrocellulose pieces are washed with 1-5 100µl volumes of 0.05% (v/v) TFA, and these volumes are concentrated to a volume of less than 100µl in a Speed-Vac (Savant;

Farmingdale, NY). These concentrates are then injected over a Vydac reverse phase Protein & Peptide C18 column (2.1mm x 100mm) installed in an Applied Biosystems (Foster City, CA) Model 130 High Performance Liquid Chromatograph (HPLC). Mobile phases used to elute peptides were: Buffer A: 0.1mM sodium phosphate, pH2.2; Buffer B: 70% acetonitrile in 0.1mM sodium phosphate, pH2.2. A 3-step gradient of 10-55% buffer B over two hours, 55-75% buffer B over 5 minutes, and 75% buffer B isocratic for 15 minutes at a flow rate of 50 µl/minute is used. Peptides are detected at 214nm, collected by hand, and then stored at -20° C.

In early digestion experiments, the PVP-40 was incompletely removed and eluted as a broad peak at 50% buffer B. Very few peptides were recovered from these runs. The PVP-40 peak was collected and then treated with an equal volume of chloroform to extract the PVP-40. The aqueous material was then reapplied to an Applied Biosystems HPLC, as above, except in this case buffer A was 0.1% TFA, buffer B was 0.1% TFA in acetonitrile, and the column used was an Applied Biosystems (Foster City, CA) reverse phase Spheri-5 RP18 column (1 mm X 50 mm). The gradient and flow rate were as above and the peaks were detected, collected and stored in the same manner.

In later digestion experiments, PVP-40 was thoroughly removed and no chloroform extraction was necessary. In order to remove the PVP-40 completely, nitrocellulose pieces are washed with many volumes of water (8 x 4 ml), checking the absorbance of the washes at 214 nm on a spectrophotometer. Also, PVP-40 is more easily removed if bands are not cut into small pieces until after PVP-40 treatment and washing. These two modifications eliminate interference problems with the PVP-40.

C. Reduction & Alkylation of Cysteine Residues

Digested protein can be reduced with β-mercaptoethanol and alkylated with ^{3}H-labelled iodoacetic acid as a method to improve identification of cysteine residues when the peptides are sequenced. The β-mercaptoethanol is added to freshly digested protein at a molar ratio of 20:1 over the assumed

concentration of sulfhydryl groups in the sample and peptides are incubated at room temperature for 30 minutes. ^{3}H-labelled iodoacetic acid is then added at a concentration of 1.1 times the concentration of β-mercaptoethanol and the peptides are incubated in darkness (covered with foil) at room temperature for 60 minutes. To stop the alklylation, more β-mercaptoethanol is added at a concentration of one-tenth of that used to reduce the peptides. Ten µl of 10% TFA is then added as usual to stop any further action of the protease. This can not be added prior to the reduction/alkylation as those reactions work best under the basic pH conditions present in the digest mixture. The reduced/alkylated protein is then concentrated in a Speed-Vac prior to separation of the peptides by HPLC, as described above.

D. N-terminal Sequencing of Proteins & Peptides

All sequencing is performed by Edman degradation on an Applied Biosystems 477A Pulsed-Liquid Phase Protein Sequencer; phenylthiohydantoin (PTH) amino acids produced by the sequencer are analyzed by an on-line Applied Biosystems 120A PTH Analyzer. Data are collected and stored on to the on-board computer of the sequencer and also on to a Digital Microvax using ACCESS*CHROM software from PE NELSON, Inc (Cupertino, CA). Sequence data is read from a chart recorder, which receives input from the PTA Analyzer, and is confirmed using quantitative data obtained from the sequencer on-board computer system. All sequence data is read independently by two operators with the aid of the data analysis system.

For peptide samples obtained as peaks off of an HPLC, the sample is loaded on to a Polybrene coated glass fiber filter (Applied Biosystems, Foster City, CA) which has been subjected to 3 pre-cycles in the sequencer. For peptides which have been reduced and alkylated, a portion of the PTA-amino acid product material from each sequencer cycle is counted in a liquid scintillation counter. For protein samples which have been electroblotted to PVDF, the band of interest is cut out and then placed above a Polybrene coated glass fiber filter, pre-cycled as above and the reaction cartridge is assembled according to manufacturer's specifications.

In order to obtain protein sequences from small amounts of sample (5-30 pmoles), the 477A conversion cycle and the 120A analyzer as described by Tempst and Riviere (*Anals. Biochem.* (1989) *183*:290).

Fragments generated from the trypsin and gluC digestion steps are presented in Fig. 2 and 3. Other proteases may be used to digest the synthase proteins, including but not limited to lysC and aspN. While the individual digest buffer conditions may be different, the protocols for digestion, peptide separation, purification, and sequencing are substantially the same as those outlined for the digestions with trypsin and gluC. Alternatively, synthases may be digested chemically using cyanogen bromide (Gross *Methods Enzymol* (1967) *11*:238-255 or Gross and Witkop *J. Am. Chem. Soc.* (1961) *83*:1510), hydroxylamine (Bornstein and Balian *Methods Enzymol.* (1977) *47*:132-745), iodosobenzoic acid (Inglis *Methods Enzymol.* (1983) *91*:324-332), or mild acid (Fontana *et al.*, *Methods Enzymol.* (1983) *91*:311-317), as described in the respective references.

Example 5. Methods to Distinguish Synthase Proteins

In this example, a method to selectively label synthase proteins with cerulenin and a method to express synthase activity in a prokaryote are described.

A. Cerulenin Labeling

1. Distinguishing Synthase I and II using Cerulenin.

Cerulenin has been shown to bind to the active sites of synthase I and synthase II. In *S. oleracea*, at a concentration of 5μM, cerulenin reacts readily with the active site of synthase I but not synthase II. Cerulenin will react readily, however with both synthase I and synthase II at concentrations above 50μM (Shimakata and Stumpf, *Proc.Nat.Acad.Sci.* (1982) *79*:5805-5812). Cerulenin shows similar reaction with synthase I and synthase II of *R. communis* as shown in Figure 1. To differentially radiolabel synthase I and synthase II for subsequent gel analysis and protein sequencing to determine

the active sites, ^{3}H-cerulenin is reacted with concentrated post-ACP-sepharose (Example 3) protein containing synthase activity at two different ^{3}H-cerulenin concentrations. The samples are then run on Laemmli SDS-PAGE and the molecular weights of the ^{3}H-labeled proteins are determined by scintillation counting. Candidate bands, containing both protein and ^{3}H-label, are digested with endoproteinase gluC and the peptides are separated and sequenced via Edman chemistry.

2. Preparation of Radiolabeled Cerulenin.

[3H]-Cerulenin (specific activity = 585 Ci/mol) was prepared from unlabeled cerulenin (Sigma) by Amersham (Arlington Heights, IL) tritium labeling service. The material is purified by HPLC.

3. Labeling Synthase I.

^{3}H-cerulenin is prepared for use by measuring out the required amount of ^{3}H-cerulenin solution (in methanol) necessary to make 1.0 mls of 5μM solution (5nmoles) into a screw-cap microfuge tube. The methanol is then evaporated under a stream of nitrogen, chasing with a diethyl ether to ensure complete evaporation of the methanol. The tube is capped and the dry ^{3}H-cerulenin is stored on ice until use (see below).

Material from ACP-Sepharose "Peak One" which contains both the 46kD and 50kD proteins is pooled and concentrated in an Amicon stirred pressure cell with a PM10 43 mm membrane. The concentrate is diluted in the cell with "zero" buffer (described in Example 3 A.2) and reconcentrated to desalt the material. The material is desalted to a phosphate concentration of approximately 20mM. Initial volume of the pooled fractions is 60-66mls and the volume of the final concentrate is 1.0ml or less, which represents a 40-fold or greater concentration.

The concentrated, desalted material is removed from the Amicon stirred cell to the microfuge tube containing the ^{3}H-cerulenin and additional "20" buffer (described in Example 3 A.2) is added to a final volume of 1 ml. The

mixture is incubated at 37°C for 20 minutes to allow complete reaction with the synthase I.

4. Labeling Synthase II.

To label Synthase II with ^{3}H-cerulenin in a mixture of synthase I and synthase II, synthase I must first be blocked with unlabeled cerulenin. Five nmoles of unlabeled cerulenin is prepared as described above for the ^{3}H-cerulenin. ^{3}H-cerulenin is prepared as above except a larger concentration is used to ensure binding to synthase II.

The concentrated enzyme is prepared as above and reacted first with 5μM unlabeled cerulenin for 20 mins at 37°C to block the synthase I from subsequent reaction with the ^{3}H-cerulenin. Following this reaction, the sample is placed in the second tube containing the higher concentration of ^{3}H-cerulenin and reacted at 37°C for 60 mins. The rest of the procedure is as described above.

5. Gel Analysis of Candidate Protein Fractions.

Laemmli-SDS-PAGE methods are as described in Example 3E, again using the reduced cross-linker formula for the gels. Gel thickness is 0.75mm. Bands from SDS-PAGE are excised and analyzed by scintillation counting to determine the molecular weight of labeled bands, and amino acid sequence of labeled fragments is determined.

6. Sequence Determination

The protein is digested with endoproteinase gluC using the method described in Example 4, with the following alteration. The digestion is carried out in solution, therefore the 5% acetonitrile added to previous digests for ease in recovery of peptides off of nitrocellulose is omitted. Reverse-phase HPLC and sequencing of peptides are as described in Example 4.

B. E. coli Expression

1. Expression Vectors.

Plasmids for expression of β-ketoacyl-ACP synthase activity in *E. coli* can be constructed using one or more *E.*

coli expression vectors. These expression vectors include pUC120, an *E. coli* expression vector based on pUC118 (Vieria and Messing, *Methods in Enzymology* (1987) *153*:3-11) with the lac region inserted in the opposite orientation and an *Nco*I site at the ATG of the lac peptide (Vieira, J. PhD. Thesis, University of Minnesota, 1988). Other expression vectors which may be used include, but are not limited to, the pET system vectors, in particular pET8C (Studier et al., *Methods in Enzymology* (1990) ed. D.V. Goedel, Vol. 185) which use a T7 RNA polymerase promoter, and pKK223 vector (Pharamacia), which utilizes a trp-lac (tac) bacterial promoter. An example of *E. coli* expression of synthase activity associated with a 50 kD protein using the pUC120 expression system is described below.

2. Constructs for Expression of Synthases

A fragment containing regions of a cDNA which encodes the 50 kD protein associated with synthase activity (Ex. 6), can be subcloned into pUC120 using commercially available linkers, restriction endonucleases, and ligase. The subcloned regions will include the coding region of the mature protein, and also possibly 5' and 3'-noncoding sequences, a transit peptide sequence, and a poly(A) tail The synthase sequences are inserted such that they are aligned in the 5' to 3' orientation with the lac transcription and translation signals. The resulting plasmid can be transformed to an appropriate strain of *E. coli* for analysis.

Constructs containing the cDNA clone for the 46 kD protein (Ex. 6) or containing the cDNAs for both the 46 kD and the 50 kD proteins can be made using the procedures and vectors described above.

3. Expression of Synthase Activity in *E.coli*

Single colonies of *E. coli* containing pUC120 or the synthase constructs in pUC120 are cultured in ECLB broth containing 300mg/L penicillin. The lac promoter is induced by the addition of 1mM IPTG. Cells are grown overnight (18 hrs) at 37°C. The overnight cultures of *E. coli* (induced and uninduced) containing pUC120 or the synthase constructs

in pUC120 are centrifuged to pellet the cells. The pelleted cells are resuspended in buffer and broken in a french press at 16,000 psi. Broken cell mixtures are centrifuged and a portion of each supernatant is applied to a G-25 Sephadex gel filtration centrifugal column (Boehringer Mannheim Biochemicals), equilibrated buffer. Columns are centrifuged and effluent is collected and used as enzyme source in the synthase assay. Synthase activity is assayed as described in Example 2. Cerulinin is also reacted with the synthase activity to distinguish synthase I and synthase II activities as described previously in Example 5A.

4. Detection of Synthase Protein in *E. coli*.

Extracts of overnight cultures of *E. coli* containing pUC120 or synthase constructs in pUC120 grown in ECLB containing 300mg/L penicillin induced with 1mM IPTG are prepared as follows. Overnight cultures grown shaking at 37°C are pelleted by centrifugation in 1.5ml Eppendorf tubes. Pellets are resuspended in SDS sample buffer (0.05M Tris-HCl, pH6.8, 1% SDS, 5% ß-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue) and boiled for 10 min. Samples are electrophoresed on a 10% polyacrylamide gel (Laemmli, *Nature* (1970) *227*:680). Gels are stained in 0.05% Coomassie Brilliant Blue, 25% isopropanol and 10% acetic acid and destained in 10% acetic acid and 10% isopropanol. Bands corresponding to synthase proteins may be detected by comparison of *E. coli* proteins produced in cells containing synthase constructs inserted in pUC120 to those containing the pUC120 vector with no insertion.

5. Expression of Synthase Activity in *E. coli* Using a T7 Promoter

A construct for the expression of the 50kD *R. communis* synthase protein in *E. coli* under control of the T7 promoter is prepared as follows. DNA sequence specifying the endonuclease restriction sites *Bam*HI and *Nde*I are inserted into the 50kD synthase cDNA clone, pCGN2765, by in vitro mutagenesis. The sequence is inserted immediately 5'

of the AAC codon specifying the asparagine N-terminal amino acid of the mature synthase protein. DNA sequence specifying the endonuclease restriction sites *Bam*HI and *Pst*I are inserted into the 50kD synthase cDNA clone immediately 3' of the TGA stop codon following the CCC codon specifying the proline C-terminal amino acid. The resulting plasmid is pCGN2773. A fragment of pCGN2773 containing the coding sequence for the mature 50kD synthase protein is subcloned by digestion with *Nde*I and *Bam*HI and ligation into *Nde*I and *Bam*HI digested pET3A, a T7 *E. coli* expression vector (Studier *et al., supra*). The resulting plasmid is pCGN2775.

Similar constructs for the expression of the 46kD *R. communis* synthase protein in *E. coli* are prepared as follows. DNA sequence specifying the endonuclease restriction sites *Bam*HI and *Nde*I are inserted into the 46kD synthase cDNA clone, 1-1A by *in vitro* mutagenesis. The sequence is inserted immediately 5' of the AAT codon specifying the asparagine amino acid located immediately to the amino end of the lysine previously identified as the N-terminal amino acid (Fig. 3) of the mature synthase protein. DNA sequence specifying the endonuclease restriction sites *Bam*HI and *Pst*I are inserted into the 46 kD synthase cDNA clone immediately 3' of the TGA stop codon which follows the TTC codon specifying the lysine C-terminal amino acid. The resulting plasmid is pCGN2774. A fragment of pCGN2774 containing the coding sequence for the mature 46 kD synthase protein is subcloned by digestion with *Nde*I and *Bam*HI and ligation into *Nde*I and *Bam*HI digested *E. coli* expression vector pET3A. The resulting plasmid is pCGN2776. A second construct for expression of the 46 kD synthase is prepared by subcloning the T7 promoter and coding sequence for the mature 46kD synthase protein from pCGN2776 by digestion with *Eco*RV and *Bgl*II (*Eco*RV and *Bgl*II restriction sites supplied by original pET3A vector) and ligation into *Bam*HI and *Eco*RV digested cloning vector pACYC184. The resulting plasmid is pCGN2777.

In addition, a construct for expression of both the 46 kD and 50 kD synthase proteins under the control of the same T7 promoter is constructed as follows. The 50 kD coding region of pCGN2773 is obtained by digestion with *Bam*HI and is ligated into *Bam*HI digested pCGN2776. This inserts the 50 kD coding sequence immediately 3' of the 46 kD coding sequence and results in plasmid pCGN2778.

The 46 kD and 50 kD constructs are transformed into *E. coli* strain BL21 (Studier *et al., supra*). Cultures are grown overnight and cells are harvested by centrifugation. The pelleted cells are resuspended to approximately 1/20 of the original volume in 20mM potassium phosphate buffer and broken using a French press apparatus. The broken cell samples are spun at approximately 12,000xg to remove cell debris and the resulting supernatant is diluted 1:1 in 40% glycerol and assayed for synthase II activity as described in Example 2 using radiolabeled C16:0-ACP as substrate in the assay. Results of these assays are presented in Table VIII.

Table VIII
Synthase Activity in *E. coli* Extracts

Construct	Protein	Specific Activity (pmol/min/µg)
pET3a		2.4
pCGN2775	50 kD	2.4
pCGN2776	46 kD	2.8
pCGN2778	46 kD + 50 kD	4.2
pACYC184		1.7
pCGN2777	46 kD	2.2
pACYC184 + pCGN2775	50 kD	2.2
pCGN2777 + pCGN2775	46 kD + 50 kD	5.2

The above results demonstrate that both the 46kD and 50kD proteins, now referred to as "synthase factor A" and "synthase factor B" respectively, are required for synthase II type activity. This is in agreement with protein purification data indicating synthase II type activity primarily in an ACP-sepharose column fraction containing both the 46 and 50kD proteins (Example 3F).

Analysis of *E. coli* extracts for the presence of synthase proteins indicates that synthase factor A comprises approximately 2% of total protein in cells transformed with pCGN2776, roughly 50 times the level of synthase factor B in the pCGN2775 cells. Further, as shown in Figure 12, the *E. coli* synthase I protein shares considerable homology at the amino acid level with plant synthase factor A and B proteins.

Additional analysis of *E. coli* cells containing the synthase constructs and control cells lacking these constructs are conducted to determine the fatty acid composition of these cells. Lipids are extracted from pelleted *E. coli* cells and fatty acids are analyzed by methanalysis and GC (gas-liquid chromatography), essentially as described by Browse *et al.* (*Anal. Biochem.* (1986) *152*:141-145). Results are presented in Table IX.

Table IX

Analysis of Fatty Acids in Transformed *E. coli*

	pET3a (Control)	pCGN2775 (50K)	pCGN2776 (46K)	pCNG2775 + pCGN2776
	%	%	%	%
12:0	3.5	3.5	3.5	3.5
14:0	5	5	3	3
16:0	27	28	21	21
16:1	22	23	10	12
Cyc17	2	2	2	1.5
18:0	0.5	0.5	5	6
18:1	37	37	51	49
18:2	3	3	2	3
18:3	0.5	0.5	0.5	0.5
20:0	--	--	0.5	0.5
20:1	tr	--	2	1.5

The above results demonstrate that the percentage of C18 fatty acids in *E. coli* increases upon expression of the plant synthase factor A protein in *E. coli*, either in the presence of absence of plant synthase factor B. When the E. coli cells are grown at 30°C instead of 37°C, as in the above experiments, an even greater effect on the *E. coli* lipids is observed. Thus, synthase II type activity may be increased by expression of the factor A protein in host cells as evidenced by increased longer chain fatty acids. In broken cells, however, increased synthase II type activity is only detectable in extracts from cells transformed with both synthase factor A and synthase factor B constructs (Table VIII). These results indicate that synthase factor A is the component which contributes the longer fatty acid substrate specificity to synthase II activity, and that factor B may or may not be required, depending on synthase factors present in the host cells.

Example 6. Isolation of Synthase Genes

In this example, the preparation of a cDNA libraries, using the methods as described in Alexander, *et al.* (*Methods in Enzymology* (1987) *154*:41-64), and the screening of the cDNA libraries for synthase cDNA clones are described.

A. R. communis cDNA Library Construction

A cDNA library may be constructed from poly(A)+ RNA isolated from *R. communis* immature endosperm from seeds collected at approximately 14-21 days post-anthesis. Total RNA is isolated from 10 g of *R. communis* immature endosperm tissue by a method described by Halling, *et al.* (*Nucl. Acids Res.* (1985) *13*:8019-8033). Total RNA is further purified by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column. The RNA is loaded onto the column in 1 ml of loading buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS), eluted with loading buffer, and collected in 500 µl fractions. Ethanol is added to the samples to precipitate the RNA. The samples are centrifuged, and the pellets resuspended in sterile

distilled water, pooled, and again precipitated in ethanol. The sample is centrifuged, and the resulting RNA is subjected to oligo(dT)-cellulose chromatography two times to enrich for poly(A)+ RNA as described by Maniatis *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1982)).

The plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene Cloning Systems; San Diego, CA), is made as follows. The polylinker of Bluescribe M13- is altered by digestion with *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent restriction sites) and annealed with synthetic complementary oligonucleotides having the sequences 5' CGGATCCACTGCAGTCTAGAGGGCCCGGGA 3' and 5' AATTTCCCGGGCCCTCTAGACTGCAGTGGATCCGAGCT 3'. These sequences are inserted to eliminate the *Eco*RI site, move the *Bam*HI site onto the opposite side of the *Sst*I (also, sometimes referred to as "*Sac*I" herein) site found in Bluescribe, and to include new restriction sites *Pst*I, *Xba*I, *Apa*I, *Sma*I. The resulting plasmid pCGN1702, is digested with *Hind*III and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *Pvu*II and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *Sst*I and homopolymer T-tails are generated on the resulting 3'-overhang sticky-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands from mRNA covalently attached to either end of the vector plasmid by their poly(A) tract. The cDNA-mRNA-vector complexes are treated with terminal transferase in the presence of deoxyguanosine

triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *Bam*HI site, is removed by *Bam*HI digestion, leaving a cDNA-mRNA-vector complex with a *Bam*HI sticky-end at one end and a G-tail at the other. This complex is cyclized using the annealed synthetic cyclizing linker, 5'-GATCCGCGGCCGCGAATTCGAGCTCCCCCCCCCC-3' and

3'-GCGCCGGCGCTTAAGCTCGA-5'

which has a *Bam*HI sticky-end and a C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5α (BRL; Gaithersburg, MD) to generate the cDNA library. The *R. communis* immature endosperm cDNA bank contains approximately $2x10^6$ clones with an average cDNA insert size of approximately 1000 base pairs.

B. Isolation of a *R. communis* cDNA Clone to the 50 kD Synthase Protein

1. Probe production.

1.1 Polymerase Chain Reactions (PCR). Amino acid sequences from two peptides from the 50 kD synthase amino acid sequence (Example 3) with low codon degeneracy, KR4 and KR16 are chosen for production of a probe for the plant 50 kD synthase cDNA. Four sets of mixed oligonucleotides are designed and synthesized for use as forward and reverse primers in the polymerase chain reaction (Saiki *et al.*, *Science* (1985) *230*:1350-1354; Oste, *Biotechniques* (1988) *6*:162-167) for the KR4 sequence, and two sets of mixed oligonucleotides are designed and synthesized for use as forward and reverse primers in the polymerase chain reaction for the KR16 sequence. All oligonucleotides are synthesized on an Applied Biosystems 380A DNA synthesizer.

The KR4 oligonucleotide groups have a redundancy of 128 and contain 20 bases of coding sequence along with flanking restriction site sequences for *Hind*III (forward primers) or *Eco*RI (reverse primers). The KR16 oligonucleotide groups have a redundancy of 384 and contain 23 bases of coding sequence along with flanking restriction site sequences for *Hind*III (forward primers) or *Eco*RI

(reverse primers). The KR4 forward and KR16 reverse primers are illustrated in Figure 4.

The KR4 oligonucleotide groups are combined so that only two polymerase chain reactions are required to account for both possible orientations of the KR4 and KR16 peptides in the synthase protein. Using the cDNA library DNA as template and the possible two combinations of the forward and reverse oligonucleotides as primers, polymerase chain reactions are performed in a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, CT) (thermocycle file 1 min. 94°C, 2 min. 42°C, 2 min rise from 42°-72°C, 3min. 72°C for 15 cycles, followed by the step cycle file without step rises, 1 min. 94°C, 2 min. 42°C, 3 min. 72°C with increasing 15 sec extensions of the 72°C step for 10 cycles, and a final 10 min. 72°C extension).

1.2 PCR Product Analysis.

1.2.1 Subcloning. The 283 bp product of the KR4 forward primer and the KR16 reverse primer reaction is gel-purified, digested with *Hind*III and *Eco*RI, and ethanol precipitated. The resulting fragment is subcloned into pCGN2015, a chloramphenicol resistant version of Bluescript KS+ (Stratagene, La Jolla, CA).

1.2.2 Construction of pCGN2015. pCGN2015 is prepared by digesting pCGN565 with *Hha*I, and the fragment containing the chloramphenicol resistance gene is excised, blunted by use of mung bean nuclease, and inserted into the *Eco*RV site of Bluescript KS- (Stratagene, La Jolla, CA) to create pCGN2008. pCGN565 is a cloning vector based on pUC12-cm (K. Buckley Ph.D. Thesis, Regulation and expression of the phi X174 lysis gene, University of California, San Diego, 1985), but contains pUC18 linkers (Yanisch-Perron, *et al.*, *Gene* (1985) *53*:103-119).

The chloramphenicol resistance gene of pCGN2008 is removed by *Eco*RI/*Hind*III digestion. After treatment with Klenow enzyme to blunt the ends, the fragment is ligated to *Dra*I digested Bluescript KS+. A clone that has the *Dra*I fragment containing ampicillin resistance replaced with the chloramphenicol resistance is chosen and named pCGN2015.

1.2.3 Clone Analysis. Minipreparation DNA (Maniatis *et al., supra*) of two clones, AG-18 and AG-32, was sequenced by Sanger dideoxy sequencing (Sanger *et al., Proc. Nat. Acad. Sci. USA* (1977) *74*:5463-5467) using the M13 universal and reverse primers. The clones were shown to have the same DNA sequence. Translation of the DNA sequence results in an amino acid sequence that contains five of the 50 kD synthase peptides (78 amino acid residues) within its 91 amino acid residues. The translated amino acid sequence was shown to have homology to the β-ketoacyl-ACP synthase I gene of *E. coli* which is encoded by fabB (Kauppinen *et al., Carlsberg Res. Commun.* (1988) *53*:357-370), and a polyketide synthase gene from *Streptomyces glaucescins* which has been shown to have homology to other β-ketoacyl synthases (Bibb *et al., EMBO J.* (1989) *8*:2727-2736).

1.3 Probe Purification. The 283 bp insert in AG-32 was amplified by PCR using the minipreparation DNA as template and the KR4 and KR16 oligonucleotides described above as primers. The resulting fragment was gel-purified for use as a probe in screening cDNA library screening.

2. Library Screen.

The *R. communis* immature endosperm cDNA bank is moved into the cloning vector lambda gt22 (Stratagene Cloning Systems, La Jolla, CA) by digestion of total cDNA with *Not*I and ligation to lambda gt22 DNA digested with *Not*I. The titer of the resulting library was approximately 1.5×10^7 pfu/ml. The library is then plated on *E. coli* strain Y1090 (Young, R.A. and Davis, R.W., *Proc. Natl. Acad. Sci. USA* (1983) *80*:1194 at a density of approximately 15,000 plaques/150 mm NZY ("NZYM" as defined in Maniatis *et al. supra*) agar plate to provide over 60,000 plaques for screening. Duplicate lifts are taken of the plaques using NEN Colony Plaque Screen filters by laying precut filters over the plates for approximately 2 minutes and then peeling them off. The phage DNA is immobilized by floating the filters on denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 min., transferring the filters to neutralizing

solution (1.5 M NaCl, 0.5 M Tris-HCI pH 8.0) for 2 min. and then to 2X SSC (1X SSC = 0.15 M NaCl; 0.015 M Na citrate) for 3 min., followed by air drying. The filters are pre-hybridized at 42°C in hybridization buffer consisting of 50% formamide, 10X Denhardts solution, 5X SSC.1% SDS, 5 mM EDTA, and 0.1 mg/ml denatured salmon sperm DNA. Filters are hybridized overnight at 42°C in the same buffer solution with added ^{32}P-labeled (Boehringer Mannheim Random Primed DNA Labeling Kit) AG-32 insert DNA described above. Filters are washed sequentially at 55°C in 1X SSC, 0.1% SDS for 25 minutes, in 0.5 X SSC, 0.1% SDS for 25 minutes, and finally in 0.1 X SSC, 0.1% SDS for 25 minutes. Filters are exposed to X-ray film at -70°C with a Dupont Cronex intensifying screen for 48 hours.

3. Clone Analysis.

Clones are detected by hybridization with the AG-32 ^{32}P-labeled DNA and plaque purified. Phage DNA is prepared from the purified clones as described by Grossberger (*NAR* (1987) *15*:6737) with the following modification. The proteinase K treatment is replaced by the addition of 10% SDS and a 10 minute incubation at room temperature. Recovered phage DNA is digested with NotI, religated at low concentration, and transformed into *E. coli* mm294 (Hanahan, *J.Mol.Biol.* (1983) *166*:557-580) cells to recover plasmids containing cDNA inserts in pCGN1703. Preliminary nucleotide sequence of the cDNA insert of the longest clone, pCGN2764 (1-3), indicates that the clone does not contain the entire coding sequence for the gene.

4. Screening for Longer Clones.

Longer cDNA clones can be obtained by screening the *R. communis* cDNA library with an oligonucleotide from the furthest 5' sequence of this clone.

A 23 base oligonucleotide, #2272, was synthesized which consists of the sequence 5' ACCAGCAACAATGCAATACCTCA 3', which is complementary to nucleotides 24-46 of cDNA clone pCGN2764. Greater than 100,000 clones from the *R. communis* embryo cDNA library in lambda gt22 are plated in

E. coli strain Y1090 as described above at a density of 20,000 plaques/150mm NZY plate. Phage are lifted onto NEN Colony/Plaque Screen filters as described above. The probe for hybridization is prepared by 5' end-labeling oligonucleotide #2272 using BRL 5X buffer and T4 kinase. Filters are prehybridized and hybridized with the probe at 37°C, and washed to remove background hybridization according to the method of Berent *et al.* (*BioTechniques* (1985) *3*:208-220). Filters are exposed to X-ray film at -70°C with a Dupont Cronex intensifying screen overnight.

12 clones are detected by hybridization to the oligonucleotide probe and are plaque-purified and recovered as pCGN1703 plasmids as described above. Minipreparation DNA (Maniatis *et al.*, supra) from each of the clones is analyzed by restriction digestion and agarose gel electrophoresis. The complete DNA sequence of the longest clone, pCGN2765 (2-8), is presented in Figure 5. DNA sequence is determined by the dideoxy-chain termination method of Sanger *et. al*, (*Proc. Natl. Acad. Sci. USA* (1977) 74:5463-5467) using the 7-Deaza-dGTP Reagent Kit with Sequenase Version 2 Enzyme (United States Biochemical Corp., Cleveland, OH). The sequence data are analyzed using the IntelliGenetics Suite of molecular programs Gel and SEQ.

C. <u>Isolation of a R. communis cDNA Clone for the 46 kD Synthase Protein</u>

1. Probe Production.

1.1 Polymerase Chain Reactions. A probe to be used in screening for a cDNA clone to the 46 kD protein can be prepared by PCR from oligonucleotides to the 46 kD peptide sequences following the procedures described above in the isolation of a cDNA to the 50 kD protein. Sequences derived from the N-terminal peptide amino acid sequence of the 46 kD protein, **KHPLMKQ**RRVVVTGMGV, (sequences used for oligo design indicated in bold letters) are used as the forward primers in four polymerase chain reactions. The reverse primers for these reactions are DNA sequences derived from 46 kD peptides KR2, **EEVNYINA**XATSTPAGDL, and

KR3, VFNDAIEALR. The forward primer has a redundancy of 128 and contains 20 bases of coding sequence along with a flanking HindIII restriction site (AAGCTT). The reverse primers have a redundancy of 128 (KR2) and 64 (KR3) and contain 20 bases of coding sequence along with flanking sequences specifying EcoRI restriction sites (GAATTC). These restriction sites are useful in cloning the product resulting from PCR. Reactions are performed in a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, CT) (step cycle file 1 min. 94°C, 2 min. 50°C, 3 min. 72°C for 15 cycles, followed by the step cycle file, 1 min. 94°C, 2 min. 42°C, 3 min. 72°C with increasing 15 sec extensions of the 72°C step for 10 cycles, and a final 10 min. 72°C extension). PCR using NTERM-B, 5'TTTAAGCTTAAPCAQCCNCTNATGAAPCA3', as forward primer, and KR2-A, 5'TTTGAATTCTTPATPTAPTTNACQTCQTC3' or KR3-A, 5'TTTGAATTCGCQTCTATNGCPTCPTTPAA3', as reverse primers, where N=A,G,C or T, Q=C or T, and P=A or G, resulted in production of prominent ~900 bp and ~330 bp bands, respectively.

1.2 Analysis of PCR products. A nitrocellulose filter containing PCR product DNA was prepared by the Southern blot technique (Maniatis *et al., supra*) The filter was probed with a synthetic oligonucleotide, Nterm11, 5'ACNCCCATNCCNGT3' prepared from the 46 kD N-terminal sequence KHPLMKQRRVVVTGMGV. Nterm-11 is derived from a different portion of the N-terminal sequence than the oligonucleotide used as forward primer in the reactions. Prehybridization, hybridization, and washing of the filter is according to Berent *et al.* (supra.), the only significant change being in the temperature used for hybridization and prehybridization, which was 25°C. Both the ~900 bp and ~330 bp bands produced by PCR as described above hybridize to this oligonucleotide. Further evidence that the ~330 bp band is a product specific to a cDNA for the 46 kD protein is obtained by repeating the PCR described above with the Nterm-B and KR3-A oligonucleotides, but using the product from the Nterm-

B/KR2-A PCR as template. The ~330 bp band is again obtained as the predominant product. The ~330 bp PCR product is digested with HindIII and EcoRI and subcloned into pCGN2015 as described above. Minipreparation DNA is prepared and DNA sequence of clones containing the ~330 bp fragment is determined as described above.

1.3 Probe Purification. The ~330 bp PCR product is gel-purified for use as probe in screening the cDNA library.

2. Library Screen.

The *R. communis* embryo cDNA library described above can be screened for a cDNA to the 46 kD synthase protein using the procedures described above in the isolation of a cDNA to the 50 kD synthase. The ~330 bp DNA fragment described above is ^{32}P-labeled using a randon primed labeling kit (Boehringer Mannheim). Greater than 60,000 clones in lambda gt22 are plated at a density of approximately 15,000/150 mm NZY plate and lifted onto NEN Colony/Plaque Screen filters as described above. Clones are detected by hybridization to the ~330 bp PCR fragment by prehybridization, hybridization, and washing as described above for screening for the cDNA to the 50 kD protein using the AG-32 fragment as probe. Clones are plaque-purified, lambda DNA is isolated, and clones are recovered as *E. coli* clones containing plasmids with inserts in pCGN1703 as described above. DNA sequence of clones is determined as described above.

3. DNA Sequence Analysis.

DNA sequence and translated amino acid sequence of a *R. communis* 46kD synthase (synthase factor A) cDNA clone, 1-1A, is presented in Figure 10. The mature protein start site is tentatively identified as the lysine residue encoded by nucleotides 365-367. Three possible translation initiation ATG codons, which would account for a transit peptide having 116, 57 or 14 amino acids, are present in the sequence 5' of the mature protein start.

Electroporation into isolated plant protoplasts of 46kD

synthase encoding constructs, which start at each of the possible translation initiation codons, is conducted to identify the codon used for initiation of translation of the 46kD synthase protein. As also observed in analysis of peptide sequences of the 50kD and 46kD synthase proteins, the translated amino acid sequences of the 46kD and 50kD synthase cDNAs demonstrate extensive homology in portions of the mature protein.

D. Isolation of Synthase Genes from Other Plant Sources

1. *B. campestris* cDNA Library Construction.

Total RNA is isolated from 5 g of *B. campestris* cv. R500 embryos obtained from seeds harvested at days 17-19 post-anthesis. RNA is extracted in 25 mls of 4 M guanidine thiocyanate buffer as described by Colbert *et al.* (*PNAS* (1983) *80*:2248-2252). Polysaccharides are removed from the RNA sample by resuspending the pellet in 6 ml of 1X TE (10 mM Tris/1 mM EDTA pH 8), adding potassium acetate to a concentration of 0.05 M, and adding one half volume of ethanol. The sample is placed on ice for 60 minutes and centrifuged for 10 minutes at 3000 x g. RNA is precipitated from the supernatant by adding sodium acetate to a concentration of 0.3 M followed by the addition of two volumes of ethanol. RNA is recovered from the sample by centrifugation at 12,000 x g for 10 minutes and yield calculated by UV spectrophotometry. Two mg of the total RNA is further purified by removing polysaccharides as described in Example 6A. The resulting RNA is enriched for poly(A)+ RNA also as described in Example 6A.

A *Brassica campestris* day 17-19 post anthesis embryo cDNA library is constructed in plasmid vector pCGN1703 using 5 µg of poly(A)+ RNA as described in Example 6A. cDNA libraries from other plant sources can be similarly prepared.

2. Genomic Library Construction

Genomic libraries can be constructed from DNA from various plant sources using commercially available vectors and published DNA isolation, fractionation, and cloning

procedures. For example, a *B. campestris* genomic library can be constructed using DNA isolated according to Scofield and Crouch (J.Biol.Chem. (1987) 262:12202-12208) that is digested with BamHI and fractionated on sucrose gradients (Maniatis *et al., supra*), and cloned into the lambda phage vector LambdaGem-11 (Promega; Madison,WI) using cloning procedures of Maniatis *et al.* (*supra*).

3. Screening cDNA and Genomic Libraries

cDNA and genomic libraries can be screened for synthase cDNA and genomic clones, respectively, using published hybridization techniques. Screening techniques are described above for screening libraries with radiolabeled oligonucleotides and longer DNA fragments. Probes for the library screening can be prepared by PCR, or from the sequence of the synthase clones provided herein. Oligonucleotides prepared from the synthase sequences may be used, as well as longer DNA fragments, up to the entire synthase clone.

For example, the *B. campestris* embryo cDNA library described above is screened for cDNA clones encoding a synthase factor B protein using a *R. communis* 50 kD synthase factor B cDNA (pCGN2764) as probe. The cDNA library is subcloned into lambda gt10 cloning vector by digestion of total cDNA with *Eco*RI and ligation to *Eco*RI digested lambda gt10. Clones are plated in an appropriate *E. coli* host and the resulting plaques lifted to duplicate nylon membrane filters. Filters are prehybridized (Maniatis *et al., supra*) overnight at 42°C. An approximately 600bp fragment of *R. communis* cDNA clone pCGN2764 is prepared by digestion with *Nco*I and gel purification of the resulting fragment. The fragment is radiolabeled (^{32}P) by nick-translation and added to prehybridized filters for overnight hybridization at 42°C.

For detection of homologous clones, hybridized filters are washed, 2 X 15 minutes, at 42°C in 2X SSC, 0.1% SDS and subjected to autoradiography. Ten clones are identified as homologous to the *R. communis* 50 kD clone and isolated by plaque purification. Phage DNA is purified, digested with

*Eco*RI and cDNAs are recovered as plasmid clones in the pCGN1703 cloning vector.

DNA sequence analysis of the 10 clones indicates that two classes of *Brassica* synthase clones having homology to the *R. communis* 50 kD synthase factor B clone were recovered, each class represented by 5 of the 10 clones. DNA and translated amino acid sequences of pCGN3248, the longest clone are presented in Figure 11A. DNA and translated amino acid sequences of 4A, a member of the second class of *B. campestris* synthase clones, are presented in Figure 11B. The sequences are approximately 94% homologous in the DNA coding region and the translated amino acid sequences are approximately 99% homologous. Comparison of pCGN3248 mature coding sequences and translated amino acid sequence to nucleic acid and translated amino acid sequences of the mature coding region of the *R. communis* synthase factor B cDNA, 2-8, indicates approximately 80% homology at the nucleic acid level and approximately 90% homology at the amino acid level.

The *Brassica* synthase factor B cDNA, pCGN3248, does not appear to be a full length clone based on comparison to the translated amino acid sequence of *R. communis* factor B clone, 2-8, and lack of an ATG initiation codon 5' of the mature protein coding region. In addition, no full length clones of the 4A sequence class were recovered in the initial screen. Full length clones are isolated by screening cDNA or genomic libraries with 5' oligonucleotides, as described above for the *R. communis* clone, or alternatively using PCR techniques.

Similar to the isolation of the Brassica synthase factor B cDNA, a clone or clones encoding a *Brassica* synthase factor A is isolated from the *B. campestris* embryo cDNA library using the *R. communis* 46kD synthase cDNA (1-1A) sequences as a probe.

To produce a probe specific for synthase factor A (46kD) sequences as opposed to the related synthase factor B (50kD), a PCR approach may be utilized. Two oligonucleotide primers specific to the *R. communis* 46kD

nucleotide sequence are synthesized from conserved synthase peptide sequences; 5' (forward) primer is a 29bp oligonucleotide corresponding to the nucleotide sequence encoding amino acids 291-300 of the RC46 sequence of Fig. 12 and 3' (reverse) primer is a 26bp oligonucleotide complementary to the nucleotide sequence encoding amino acids 396-404 of the RC46 sequence of Fig. 12. The above 29bp and 26bp primers are used in PCR to produce a 340bp synthase fragment using DNA from the *B. campestris* cDNA library as template. Due to homology of the *B. campestris* synthase factor B clone, the PCR generated fragment was also presumed to contain factor B sequences. Taking advantage of two *Dde*I sites in this region in both the *R. communis* and *Brassica* factor B sequences and the lack of these sites in the *R. communis* synthase factor A sequence, the PCR product is digested with *Dde*I. This digestion results in the 340bp synthase factor A sequence and two smaller fragments of approximately 270 and 70 base pairs which represent digested synthase factor B sequences.

Southern blot analysis of the resulting DNA using a 21bp oligonucleotide probe complementary to the nucleotide sequence encoding amino acids 376-382 of the RC46 sequence of Fig. 12, a region where the factor A and factor B sequences are not homologous, confirms that the 340bp fragment is homologous to the RC46 probe, while the smaller synthase factor B fragments are not homologous. The 340bp factor A specific fragment is gel-purified, radiolabeled and used to screen the *B. campestris* embryo cDNA library as previously described. Thirty four candidates for Brassica factor A were identified and 32 were confirmed as representing synthase factor A by PCR with the above-described 29bp (5') and 21bp (3') primers.

To identify the longest clones among the *Brassica* synthase factor A clones, PCR is conducted using a forward primer specific to pCGN1703 vector sequences near the cDNA 5' end cloning site and a 26bp synthetic oligonucleotide complementary to the nucleotide sequence encoding amino acids 38-46 of the RC46 sequence of Fig. 12. Six of the

Brassica synthase factor A clones yielded distinct bands from the PCR and were selected for further plaque purification and sequence analysis.

Example 7. Synthase Constructs in Plants

In this example, constructs containing *R. communis* synthase gene(s) suitable for plant transformation are described.

A. Expression Cassettes

Expression cassettes utilizing 5'-upstream sequences and 3'-downstream sequences of genes preferentially expressed during seed development can be constructed from isolated DNA sequences of genes with an appropriate expression pattern. Examples of genes which are expressed during seed development in *Brassica* are a napin gene, 1-2, and an ACP gene, Bcg4-4, both described in European Patent Publication EP 0 255 378, and a Bce4 gene, as described below. The napin gene encodes a seed storage protein that is preferentially expressed in immature embryos which are actively producing storage proteins. The ACP gene encodes a protein which is an integral factor in the synthesis of fatty acids in the developing embryo and is preferentially expressed during fatty acid synthesis. Bce4 is a gene that produces a protein of unknown function that is preferentially expressed early in embryo development, at about 15-19 days post-anthesis, and is also detectable as early as 11 days post-anthesis. The sequence of Bce4 is shown in Figure 7. The 5' and 3' regulatory sequences are obtainable from a genomic library of *B. campestris*, which can be constructed as described in Example 6D.

DNA sequences that control the expression of these genes can be isolated and sufficient portions of the 5' and 3' regulatory regions combined such that a synthase gene inserted between these sequences will be preferentially expressed early in seed development. This expression pattern will allow the synthase gene to affect fatty acid synthesis, which also occurs early in seed development. For example, a 1.45 kb *Xho*I fragment containing 5' sequence

and a 1.5 kb *SstI/BglII* fragment containing 3' sequence of the Bcg4-4 ACP gene can be combined in an ACP expression cassette using a variety of available DNA manipulation techniques. Similarly, a napin expression cassette can be prepared that contains approximately 1.725 kb of 5' sequence from an EcoRV site to immediately before the ATG start codon and approximately 1.25 kb of 3' sequence from an XhoI site approximately 18 bases past the TAG stop codon to a 3' HindIII site of a 1-2 napin gene. A Bce4 expression cassette can be made by combining approximately 7.4 kb of 5' DNA sequence from an upstream *PstI* site to immediately before the ATG start codon with approximately 1.9 kb of 3' sequences from immediately after the TAA stop codon to a 3' *PstI* site.

Variations can be made in these expression cassettes such as increasing or decreasing the amounts of 5' and 3' sequences, combining the 5' sequences of one gene with the 3' sequences of a different gene (for example using the 1.3 kb 5' sequences of napin 1-2 with the 1.5 kb 3' sequences of ACP Bcg4-4 in an expression cassette), or using other available 3' regulatory sequences, as long as these variations result in expression cassettes that allow for expression of the inserted synthase gene at an appropriate time during seed development.

1. Napin Seed Specific Expression Cassettes

Napin 1-2 pCGN1808 Expression Cassette

An expression cassette utilizing 5' upstream sequences and 3' downstream sequences obtainable from *B. campestris* napin gene can be constructed as follows.

A 2.7 kb *XhoI* fragment of napin 1-2 (*See*, Figure 2 of EP 0 255 378, published February 3, 1988) containing 5' upstream sequences is subcloned into pCGN789 (a pUC based vector with the normal polylinker replaced by the synthetic linker which encodes the restriction digestion sites *EcoRI*, *SalI*, *BglII*, *PstI*, *XhoI*, *BamHI*, *HindIII*) and results in pCGN940. The majority of the napin coding region of pCGN940 was deleted by digestion with *SalI* and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was

used in an *in vitro* mutagenesis reaction (Adelman *et al.*, *DNA* (1983) *2*:183-193) using a synthetic oligonucleotide which inserted *Eco*RV and *Nco*I restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

A 1.7 kb promoter fragment was subcloned from pCGN1801 by partial digestion with *Eco*RV and ligation to pCGN786 (a pCGN566 (polylinker in opposite orientation as pCGN565 described in Example 6 B.1) chloramphenicol based vector with the synthetic linker described above in place of the normal polylinker) cut with *Eco*RI and blunted by filling in with DNA Polymerase I Klenow fragment to create pCGN1802.

A 2.1 kb *Sal*I fragment of napin 1-2 containing 3' downstream sequences is subcloned into pCGN789 (described above) and results in pCGN941. pCGN941 is digested with *Xho*I and *Hind*III and the resulting approximately 1.6 kb of napin 3' sequences are inserted into *Xho*I-*Hind*III digested pCGN1802 to result in pCGN1803. In order to remove a 326 nucleotide *Hind*III fragment inserted opposite to its natural orientation, as a result of the fact that there are 2 *Hind*III sites in pCGN1803, the pCGN1803 is digested with *Hind*III and religated. Following religation, a clone is selected which now contains only 1.25 kb of the original 1.6 napin 3' sequence. This clone, pCGN1808 is the napin 1-2 expression cassette and contains 1.725 kb of napin promoter sequences and 1.265 kb of napin 3' sequence with the unique cloning sites *Sal*I, *Bgl*I, *Pst*I and *Xho*I in between.

Napin 1-2 pCGN3223 Expression Cassette

Alternatively, pCGN1808 may be modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, *supra*). Synthetic oligonucleotides containing *Kpn*I, *Not*I and *Hind*III restriction sites are annealed and ligated at the unique *Hind*III site of pCGN1808, such that

only one *Hin*dIII site is recovered. The resulting plasmid, pCGN3200 contains unique *Hin*dIII, *Not*I and *Kpn*I restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *Hin*dIII and *Sac*I and ligation to *Hin*dIII and *Sac*I digested pIC19R (Marsh, *et al.* (1984) *Gene 32*:481-485) to make pCGN3212. The extreme 5'-sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *Sac*I site and the junction of the napin 5'-promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains *Cla*I, *Hin*dIII, *Not*I, and *Kpn*I restriction sites as well as nucleotides 408-423 of the napin 5'-sequence (from the *Eco*RV site) and the reverse primer contains the complement to napin sequences 718-739 which include the unique *Sac*I site in the 5'-promoter. The PCR was performed using a Perkin Elmer/Cetus thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene 19*:259-268) and digested with *Hin*cII to give pCGN3217. Sequence of pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The resulting expression cassette pCGN3221, is digested with *Hin*dIII and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hin*dIII. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *Hin*dIII, *Not*I and *Kpn*I restriction sites and unique *Sal*I, *Bgl*II, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions.

2. Bce4 Expression Cassette

An expression cassette for seed specific expression can also be constructed from Bce4 gene sequences, such as those represented in Figure 7. Genomic clones having regulatory sequences of the Bce4 gene may be isolated from a *Brassica campestris* genomic library using Bce4 sequences as probe. For example, an approximately 20 kb *Bam*HI fragment is isolated and designated as clone P1C1. The approximately 20 kb insert of clone P1C1 is released by *Bam*HI digestion and inserted into the *Bam*HI site of the binary vector pCGN1547 (see below), producing pCGN1853. The *Pst*I fragment of pCGN1853, containing the Bce4 gene, is inserted into the *Pst*I site of pUC18 (Norrander, *et al.*, (1983) *supra*), producing pCGN1857. The plasmid pCGN1857 was deposited with the ATCC, Rockville, MD on March 9, 1990, accession number 68251. The *Cla*I fragment of pCGN1857, containing the Bce4 gene is ligated into *Cla*I digested Bluescript KS+ (Stratagene; La Jolla, CA), producing pCGN1864. Single stranded DNA is made from pCGN1864 and altered by *in vitro* mutagenesis as described by Adelman *et al.* (*DNA* (1983) *2*:183-193) using oligonucleotides having homology to Bce4 sequences 5' and 3' of the translated start and stop codons and also coding for restriction digest sites. The resulting plasmid, pCGN1866, contains *Xho*I and *Bam*HI sites (from BCE45P) immediately 5' to the Bce4 start codon and *Bam*HI and *Sma*I sites (from BCE43P) immediately 3' to the Bce4 stop codon. The *Cla*I fragment of pCGN1866, containing the mutagenized sequences, is inserted into the *Cla*I site of pCGN2016 (described below), producing pCGN1866C. The *Cla*I fragment of pCGN1866C is used to replace the corresponding wild-type *Cla*I fragment of pCGN1867 (described below) to produce pCGN1868. Bce4 coding sequences are removed by digestion of pCGN1868 with *Bam*HI and recircularization of the plasmid to produce pCGN1870. The Bce4 expression cassette, pCGN1870, contains 7.4 kb of 5' regulatory sequence and 1.9 kb of 3' regulatory sequence derived from the Bce4 genomic

clone separated by the cloning sites, *Xho*I, *Bam*HI, and *Sma*I.

pCGN1867

The *Bam*HI and *Sma*I sites of pUC18 (Norrander *et al.*, (1983) *supra*) are removed by *Bam*HI-*Sma*I digestion and recircularization of the plasmid, without repair of the ends, to produce pCGN1862. The *Pst*I fragment of pCGN1857, containing the Bce4 gene, is inserted into the *Pst*I site of pCGN1862 to produce pCGN1867.

pCGN2016

The multiple cloning sites of pUC12-Cm (Buckley, K., Ph.D. Thesis, UCSD, CA (1985)) are replaced by those of pUC18 to produce pCGN565. The *Hha*I fragment of pCGN565, containing the chloramphenicol resistance gene is excised, blunted by use of mung bean nuclease, and inserted into the *Eco*RV site of Bluescript KS- (Stratagene; La Jolla, CA) to create pCGN2008. The chloramphenicol resistance gene of pCGN2008 is removed by *Eco*RI-*Hin*dIII digestion. After treatment with Klenow enzyme to blunt the ends, the fragment carrying the chloramphenicol resistance gene is inserted into the *Dra*I site of Bluescript KS-, replacing the ampicillin resistance gene of Bluescript KS-, to produce pCGN2016.

B. Synthase Constructs in Plants

1. Insertion of Synthase Gene into Expression Cassettes.

Synthase cDNA sequences from isolated cDNA clones can be inserted in the expression cassettes in either the sense or anti-sense orientation using a variety of DNA manipulation techniques. If convenient restriction sites are present in the synthase clones, they may be inserted into the expression cassette by digesting with the restriction endonucleases and ligation into the cassette that has been digested at one or more of the available cloning sites. If convenient restriction sites are not available in the clones, the DNA of either the cassette or the synthase gene(s), can be modified in a variety of ways to facilitate cloning of the synthase gene(s) into the cassette. Examples of methods to modify the DNA include by

PCR, synthetic linker or adaptor ligation, *in vitro* site-directed mutagenesis (Adelman *et al.*, supra), filling in or cutting back of overhanging 5' or 3' ends, and the like. These and other methods of manipulating DNA are well known to those of ordinary skill in the art.

For example, the *R. communis* synthase factor A cDNA, 1-1A, is altered by in vitro mutagenesis to insert a *Bam*HI restriction site at the 5' end of the cDNA insert and *Xho*I and *Sma*I sites immediately 3' of the translation stop codon. The resulting construct, pCGN2781, is digested with *Bam*HI and *Xho*I and ligated into *Bgl*II and *Xho*I digested pCGN3223, the above described napin expression cassette, resulting in pCGN2785.

The *R. communis* synthase factor B cDNA, pCGN2765 (2-8), is altered by in vitro mutagenesis to insert a *Bam*HI restriction site at the 5' end of the cDNA insert and *Xho*I and *Sma*I sites immediately 3' of the translation stop codon. The resulting construct, pCGN2783, is digested with *Bam*HI and *Xho*I and ligated into *Bgl*II and *Xho*I digested pCGN3223, the above described napin expression cassette, resulting in pCGN2786.

Similarly, synthase constructs for expression of anti sense sequences may be prepared. For example, the *Brassica* campestris synthase factor B cDNA clone, pCGN3248, is mutagenized to insert SmaI, BglII and *Sal*I restriction sites approximately 200 bases 3' of the translation stop signal, resulting in pCGN3255. pCGN3255 is digested at the factor B cDNA internal *Sal*I site located approximately 140 bases in from the 5' end of the cDNA and at the 3' *Bgl*II site inserted by mutagenesis. The resulting synthase factor B cDNA fragment is ligated into *Bgl*II and *Sal*I digested pCGN3223, the above described napin expression cassette, resulting in antisense construct pCGN3257. Thus, transcription of the *Brassica* synthase factor B sequence from the napin promoter will result in production of an mRNA strand that is complementary to that of the endogenous *Brassica* synthase factor B gene.

A similar *Brassica* synthase factor B antisense construct is prepared in a Bce4 expression cassette. pCGN3255 (described above) is digested with *Sal*I to yield a synthase factor B gene fragment. This fragment is ligated into *Xho*I digested Bce4 expression cassette, pCGN1870 (described above), resulting in antisense construct pCGN3260.

2. Binary Vectors for Plant Transformation

The fragment containing the synthase gene in the expression cassette, 5' sequences/synthase/3' sequences, can be cloned into a binary vector such as described by McBride and Summerfelt (*Pl.Mol.Biol.* (1990) *14*:269-276) for *Agrobacterium* transformation. Other binary vectors are known in the art and may also be used for synthase cassettes.

For example, the antisense *Brassica* synthase factor B construct in a napin expression cassette, pCGN3257 is digested with *Asp*718 (same recognition sequence as *Kpn*I) and cloned into *Asp*718 digested pCGN1578 (McBride and Summerfelt, *supra*) yielding binary construct pCGN3259. The antisense *Brassica* synthase factor B construct in a Bce4 expression cassette, pCGN3260 is digested with *Pst*I and cloned into *Pst*I digested pCGN1578 (McBride and Summerfelt, *supra*) yielding binary construct pCGN3261.

Similarly, the *R. communis* synthase factors A and B constructs described above are ligated into pCGN1557 (McBride and Summerfelt, *supra*) or a similar construct to yield binary vectors for plant transformation with the synthase factor expression constructs.

The binary vector containing the expression cassette and the synthase gene is transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood, *et al.*, *J. Bacteriol.* (1986) *168*:1291-1301) as per the method of Holsters, *et al.*, (*Mol. Gen. Genet.* (1978) *163*:181-187) for plant transformation as described in Example 9.

3. Analysis of Transformed Plants

Transformed *Brassica campestris* cv. Tobin plants are obtained, as described for *B. napus* in Example 9, by cocultivation with *Agrobacterium* cells containing the binary construct, pCGN3259. Oil analysis of mature single seeds from the resulting plants (Browse *et al.*, *supra*) reveals seed oil with reduced stearate content, thus supporting the contribution of synthase factor B to synthase II type activity.

4. Other Methods of Plant Transformation

The binary vectors described above are useful for *Agrobacterium*-mediated plant transformation methods. Other methods for plant transformation, such as the DNA-bombardment technique described in Example 9B, and electroporation may be used as well.

5. Constructs Containing More than One Synthase Gene.

If more than one synthase gene is required to obtain an optimum effect in plants, the genes may be expressed under regulation of two different promoters that are preferentially expressed in developing seeds, such as the napin, ACP, and Bce4 sequences described above, and introduced into plants in the same binary vector, or introduced simultaneously in different binary vectors. Use of more than one binary vector would require the use of additional selectable markers to allow for selection of both genes. Examples of selectable markers that can be used include the nptII gene for kanamycin resistance, which is used in the binary vectors of McBride and Summerfelt (*supra*), the nitrilase gene, bxn, that confers resistance to the herbicide bromoxynil, described by Stalker *et al.* (*Science* (1988) *242*:419-423), and a gene that confers resistance to the antibiotic hygromycin, (van den Elzen *et al.*, *Pl.Mol.Biol.* (1985) 5:299-302). Other selectable markers are also known and can be used in binary vectors for plant transformation. Alternatively, the genes can be introduced into the plant sequentially by transforming a plant expressing one of the desired genes with a construct containing a second desired gene. This method would also

require the use of a different selectable marker for selection of the second gene.

Another alternative for obtaining expression of more than one synthase gene in a transformed plant is to produce by the methods described above, different plants, each of which is expressing one of the synthase genes. A plant expressing both genes can be obtained by back-crossing or other plant breeding techniques.

Example 8. Plants Transformed With Synthase and Desaturase

In this example constructs containing a desaturase gene isolated from *C. tinctorius* are described. The complete cDNA sequence of the *C. tinctorius* desaturase clone, pCGN2754, is presented in Figure 8.

A. Desaturase Gene in an ACP Expression Cassette

The preparation of an ACP expression cassette containing *C. tinctorius* Δ-9 desaturase in a binary vector suitable for plant transformation is described.

An expression cassette utilizing 5'-upstream sequences and 3'-downstream sequences obtainable from *B. campestris* ACP gene can be constructed as follows.

A 1.45kb *Xho*I fragment of Bcg 4-4 containing 5'-upstream sequences is subcloned into the cloning/sequencing vector Bluescript+ (Stratagene Cloning Systems, San Diego, CA). The resulting construct, pCGN1941, is digested with *Xho*I and ligated to a chloramphenicol resistant Bluescript M13+ vector, pCGN2015 digested with *Xho*I. pCGN2015 described in Example 6. This alters the antibiotic resistance of the plasmid from penicillin resistance to chloramphenicol resistance. The chloramphenicol resistant plasmid is pCGN1953.

3'-sequences of Bcg 4-4 are contained on an *Sst*I/BglII fragment cloned in the *Sst*I/*Bam*HI sites of M13 Bluescript+ vector. This plasmid is named pCGN1940. pCGN1940 is modified by *in vitro* site-directed mutagenesis (Adelman *et al.*, *DNA* (1983) *2*:183-193) using the synthetic oligonucleotide 5'-CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTAAGAG-

3' to insert *Sma*I and *Pst*I restriction sites immediately following the stop codon of the reading frame for the ACP gene 18 nucleotides from the *Sst*I site. The 3'-noncoding sequences from this modified plasmid, pCGN1950, are moved as a *PsI-Sma*I fragment into pCGN1953 cut with *Pst*I and *Sma*I. The resulting plasmid pCGN1977 comprises the ACP expression cassette with the unique restriction sites *Eco*RV, *Eco*RI and *Pst*I available between the 1.45kb 5' and 1.5 kb of 3'-noncoding sequences for the cloning of genes to be expressed under regulation of these ACP gene regions.

Desaturase cDNA sequences from pCGN2754 are inserted in the ACP expression cassette, pCGN1977, as follows. pCGN2754 is digested with *Hind*III (located 160 nucleotides upstream of the start codon) and *Asp*718 located in the polylinker outside the poly(A) tails. The fragment containing the coding region for desaturase was blunt-ended using DNA polymerase I and ligated to pCGN1977 digested with *Eco*RV. A clone containing the desaturase sequences in the sense orientation with respect to the ACP promoter is selected and called pCGN1895. The fragment containing the pCGN1895 expression sequences ACP 5'/desaturase/ACP 3' is cloned into a binary vector pCGN1557 (described below) for *Agrobacterium* transformation by digestion with *Asp*718 and *Xba*I and ligation to pCGN1557 digested with *Asp*718 and *Xba*I. The resulting binary vector is called pCGN1898.

B. Desaturase in an Anti-Sense Construct

Cassettes for transcription of antisense constructs include, but are not limited to the seed preferential expression cassettes described in Example 7. An antisense construct is described below which allows for constitutive transcription of a *B. campestris* desaturase cDNA clone in the 5' to 3' orientation of transcription such that the mRNA strand produced is complementary to that of the endogenous desaturase gene.

1. Isolation of *B. campestris* Desaturase cDNA.

cDNA clones for desaturase are isolated from the *B. campestris* cDNA library described in Example 6 D.1.

Partial DNA sequence of two clones, pCGN3235 and pCGN3236, are presented in Figure 9A and 9B, respectively. Initial DNA sequence analysis of the 3' regions of these clones indicates that pCGN3235 and pCGN3236 are cDNA clones from the same gene. pCGN3236 is a shorter clone than pCGN3235, which appears to contain the entire coding region of the *B. campestris* desaturase gene.

2. Binary Vector Construction.

The *Kpn*I, *Bam*HI, and *Xba*I sites of binary vector pCGN1559 (McBride and Summerfelt, *Pl.Mol.Biol.* (1990) *14*: 269-276) are removed by *Asp*718/*Xba*I digestion followed by blunting the ends and recircularization to produce pCGP67. The 1.84 kb *Pst*I/*Hind*III fragment of pCGN986 containing the 35S promoter-tml3' cassette is inserted into *Pst*I/*Hind*III digested pCGP67 to produce pCGP291.

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an *Alu*I fragment (bp 7144-7734) (Gardner *et. al.*, *Nucl.Acids Res.* (1981) *9*:2871-2888) into the *Hinc*II site of M13mp7 (Messing, *et. al.*, *Nucl.Acids Res.* (1981) *9*:309-321) to create C614. An *Eco*RI digest of C614 produced the *Eco*RI fragment from C614 containing the 35S promoter which is cloned into the *Eco*RI site of pUC8 (Vieira and Messing, *Gene* (1982) *19*:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II promoter fragment from pCGN147. This fragment is cloned into the *Bgl*II site of pCGN528 so that the *Bgl*II site is proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson

et. al., Mol. Gen. Genet. (1979) *177*:65) with *Hin*dIII-*Bam*HI and inserting the *Hin*dIII-*Bam*HI fragment containing the kanamycin gene into the *Hin*dIII-*Bam*HI sites in the tetracycline gene of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) *134*:1141-1156). pCGN526 was made by inserting the *Bam*HI fragment 19 of pTiA6 (Thomashow *et. al., Cell* (1980) *19*:729-739), modified with *Xho*I linkers inserted into the *Sma*I site, into the *Bam*HI site of pCGN525. pCGN528 is obtained by deleting the small *Xho*I fragment from pCGN526 by digesting with *Xho*I and religating.

pCGN149a is made by cloning the *Bam*HI-kanamycin gene fragment from pMB9KanXXI into the *Bam*HI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene* (1982) *19*:259-268) which has the *Xho*I site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in *Agrobacterium*.

pCGN149a is digested with *Hin*dIII and *Bam*HI and ligated to pUC8 digested with *Hin*dIII and *Bam*HI to produce pCGN169. This removes the Tn903 kanamycin marker. pCGN565 (described in Example 6) and pCGN169 are both digested with *Hin*dIII and *Pst*I and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *Pst*I site, Jorgenson *et. al.*, (1979), *supra*). A 3'-regulatory region is added to pCGN203 from pCGN204, an *Eco*RI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Yanisch-Perron, *et al., Gene* (1985) *33*:103-119) by digestion with *Hin*dIII and *Pst*I and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from the *Bam*19 T-DNA fragment (Thomashow *et al.*, (1980) *supra*) as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823, numbering as in Barker *et al., Plant Mol. Biol.* (1982) *2*:335-350) and combined with the pACYC184 (Chang and Cohen (1978), *supra*) origin of replication as an *Eco*RI-*Hin*dIII fragment and a gentamycin resistance marker (from plasmid

pLB41), obtained from D. Figurski) as a *Bam*HI-*Hin*dIII fragment to produce pCGN417.

The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*19 fragment) is changed to a *Sac*I site using linkers and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 is changed to an *Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I fragment containing the tml 3' regulatory sequences is joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give pCGN975. The small part of the Tn5 kanamycin resistance gene is deleted from the 3'-end of the CaMV 35S promoter by digestion with *Sal*I and *Bgl*II, blunting the ends and ligation with *Sal*I linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two *Sal*I sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

3. Insertion of Desaturase Sequence.

The 1.6 kb *Xba*I fragment from a *B. campestris* desaturase cDNA clone, pCGN3235, which contains the desaturase cDNA is inserted in the antisense orientation into the XbaI site of pCGP291 to produce pCGN3234. A desaturase construct is transformed into a desired plant host using any appropriate method, such as described in Example 9.

C. Synthase and Desaturase Constructs in Plants

Plants containing both a synthase construct as described in Example 7 and a desaturase construct, such as those described above, may be prepared using any appropriate transformation method, such as those described in Example 9. The constructs may be combined in a common binary vector, when *Agrobacterium* is used for plant transformation, or introduced into a plant simultaneously on different binary vectors using different selectable markers. Also, plants containing synthase and desaturase constructs may be prepared by retransformation of a plant that contains one of the desired sequences, with a construct containing the other desired sequence.

Another alternative for obtaining expression of more than one synthase gene or genes and a desaturase gene in a transformed plant is to produce by the methods described above, different plants, each of which is expressing one of the desired genes. A plant expressing all desired genes can be obtained by back-crossing or other plant breeding techniques.

Example 9. Plant Transformation

In this example, an *Agrobacterium*-mediated plant transformation is described and *Brassica napus* is exemplified. Also, a DNA-bombardment plant transformation is described and peanut transformation is exemplified.

A. Transformation of *B. napus*

Seeds of *Brassica napus* cv. Delta are soaked in 95% ethanol for 2 min, surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water. Seeds are then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco) supplemented with pyrodoxine (50 µg/l), nicotinic acid (50 µg/l), glycine (200 µg/l), and 0.6% Phytagar (Gibco) pH 5.8. Seeds are germinated in a culture room at 22°C in a 16 h photoperiod with cool fluorescent and red light of intensity approximately 65 µEinsteins per square meter per second ($\mu Em^{-2}s^{-1}$).

Hypocotyls are excised from 7 day old seedlings, cut into pieces approximately 4 mm in length, and plated on feeder plates (Horsch et al. 1985). Feeder plates are prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100x25 mm) containing about 30 ml MS salt base (Carolina Biological) 100 mg/l inositol, 1.3 mg/l thiamine-HCl, 200 mg KH_2PO_4 with 3% sucrose, 2,4-D (1.0 mg/l), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (MSO/1/0 medium). A sterile filter paper disc (Whatman 3 mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10 ml of

culture into 100 ml fresh MS medium as described for the feeder plates with 2,4-D (0.2 mg/l), Kinetin (0.1 mg/l). All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30 $\mu Em^{-2}s^{-1}$ to 65 $\mu EM^{-2}s^{-1}$.

Single colonies of *A. tumefaciens* strain EHA101 containing a binary plasmid are transferred to 5 ml MG/L broth and grown overnight at 30°C. Per liter, MG/L broth contains 5g mannitol, 1 g L-glutamic acid or 1.15 g sodium glutamate, 0.25 g kH_2PO_4, 0.10 g NaCL, 0.10 g $MGSO_4 \cdot 7H_20$, 1 mg biotin, 5 g tryptone, and 2.5 g yeast extract, and the broth is adjusted to pH 7.0. Hypocotyl explants are immersed in 7-12 ml MG/L broth with bacteria diluted to $1x10^8$ bacteria/ml and after 10-20 min. are placed onto feeder plates. After 48 h of co-incubation with *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500 mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim) at concentrations of 25 mg/l.

After 3-7 days in culture at 65 $\mu Em^{-2}s^{-1}$ to 75 $\mu Em^{-2}s^{-1}$ continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3 mg/l benzylaminopurine, 1 mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500 mg/l) and kanamycin sulfate (25 mg/l). Hypocotyl explants are subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1 cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300 mg/l), kanamycin sulfate (50 mg/l) and 0.6% Phytagar) and placed in a culture room with conditions as described for seed germination. After 2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2

mg/l indolebutyric acid, 50 mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPT II activity.

B. Peanut Transformation

DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter region, a gene of interest, and a termination region, into a plant genome via particle bombardment as described in European Patent Application 332 855 and in co-pending application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging from 0.5μM-3μM are coated with DNA of an expression cassette. This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from cotyledonary explants, shoot meristems, immature leaflets, or anthers.

The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics™ particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20 cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of nylon nets with mesh ranging from 10μM to 300μM.

Following bombardment, plants may be regenerated following the method of Atreya, *et al.*, (*Plant Science Letters* (1984) *34*:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and Skoog, *Physio. Plant.* (1962) *15*:473) (MS plus 2.0 mg.l 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25 ± 2°C and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m^2). On the 10th day of culture, the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days are and finally moved to greenhouse.

The putative transgenic shoots are rooted. Integration of exogenous DNA into the plant genome may be confirmed by various methods known to those skilled in the art.

The above results demonstrate the ability to obtain protein preparations with synthases activities, especially synthase I or synthase II, isolate DNA sequences related to such protein preparations and manipulate them. In this manner, the production of transcription constructs and expression cassettes can be produced which allow for production, especially differentiated cell products, or inhibition of plant synthases. Thus, the phenotype of a particular plant may be modified.

A purified *R. communis* synthase is provided and used to obtain nucleic acid sequences. From the protein or the sequences other plant synthases may be obtained.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by referenced to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

What is claimed is:

1. A plant β-ketoacyl synthase protein preparation obtainable from *R. communis* having preferential activity towards shorter chain length acyl-ACPs as compared to longer chain length acyl-ACPs.

2. The protein preparation of Claim 1 having a specific activity of at least 16 μmol/min/mg protein.

3. A plant β-ketoacyl synthase protein preparation having preferential activity towards longer chain length acyl-ACPs as compared to shorter chain length acyl-ACPs.

4. The protein preparation of Claim 3 having a specific activity of at least 1.7 μmol/min/mg protein.

5. The protein preparation of Claim 3 wherein said protein is obtainable from *R. communis*.

6. A plant β-ketoacyl synthase protein preparation prepared according to a method comprising the steps of:

a. fractionating a homogenate of R. communis immature endosperm with an ammonium sulfate solution of about 40% weight per volume and recovering supernatant;

b. contacting said supernatant with a higher concentration, as compared to step (a) of ammonium sulfate sufficient to precipitate protein having active synthase protein;

c. resuspending the precipitate recovered in step (b) and subjecting the resulting solution to Reactive Green-19 Agarose chromatography and eluting proteins having synthase activity in a high salt wash;

d. absorbing partially desalted protein fractions prepared from step (c) onto an ACP-Sepharose column;

e. eluting fractions having plant synthase activity with a gradient of 100-250 mM potassium phosphate buffer.

7. The protein preparation of Claim 6 further comprising the step of applying a first major fraction eluted from step (e) to SDS-PAGE analysis and observing at least two major bands of about 50 kD and 46 kD.

8. The protein preparation of Claim 7 wherein said 50 kD protein preparation comprises amino acid sequence shown in Fig. 6.

9. The protein preparation of Claim 6 further comprising the step of applying a later major fraction eluted from step (e) to SDS-PAGE analysis and observing at least one major band of about 50 kD.

10. A cDNA sequence encoding at least a portion of the 50 kD protein of Claim 7.

11. A cDNA sequence encoding at least a portion of the 46 kD protein of Claim 7.

12. A cDNA sequence encoding at least a portion of the 50 kD protein of Claim 9.

13. A nucleic acid sequence encoding a β-ketoacyl synthase obtainable from the cDNA of any one of Claims 10-12 joined to a heterolgous sequence.

14. A cDNA sequence encoding at least a portion of a β-ketoacyl-ACP synthase obtainable from the cDNA of any one of Claims 10-12.

15. A method of catalyzing the condensation reaction between an acyl-ACP having a chain length of C2 to C16 and malonyl-ACP comprising the steps of

contacting said acyl-ACP and said maloynl-ACP with a β-ketoacyl synthase protein preparation obtainable from *R. communis*, wherein said synthase is external to a *R. communis* cell, under conditions which permit the condensation of said acyl-ACP and malonyl-ACP.

16. The method of Claim 15 wherein said acyl-ACP has a chain length of C2 to C14 and said synthase has a preferential activity towards shorter acyl-ACP fatty acids as compared to longer acyl-ACP fatty acids.

17. The method of Claim 15 wherein said acyl-ACP has a chain length of C14 or C16 and said synthase has a preferential activity towards longer acyl-ACP fatty acids as compared to shorter acyl-ACP fatty acids.

18. The method of Claim 15 wherein said contacting occurs *in vitro*.

19. The method of Claim 15 wherein said contacting occurs *in vivo*.

20. The method of Claim 19 further comprising growing a plant cell having integrated in its genome a expression construct capable of expressing a plant synthase.

21. A recombinant DNA construct comprising at least a portion of a plant synthase protein encoding sequence.

22. The recombinant DNA construct of Claim 21, wherein said plant synthase protein is synthase factor A.

23. The recombinant DNA construct of Claim 21, wherein said plant synthase protein is synthase factor B.

24. The construct of Claim 21 encoding a biologically active plant synthase.

25. The construct of Claim 21 wherein said sequence is joined to a second nucleic acid sequence which is not naturally joined to said first sequence.

26. The construct of Claim 21 wherein said sequence is joined to a label.

27. The construct of Claim 21 wherein said sequence encodes a precursor plant synthase protein.

28. The construct of Claim 21 comprising, in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in a host cell and said sequence.

29. The construct of Claim 28 wherein said sequence is a sense sequence.

30. The construct of Claim 28 wherein said sequence is an anti-sense sequence.

31. The construct of Claim 28 further comprising, a translational regulatory region functional in a host cell immediately 5' to said sequence and a transcriptional/ translational termination regulatory region 3' to said sequence.

32. The construct of Claim 21 comprising an expression cassette capable of producing a plant synthase protein in a host cell comprising, in the 5' to 3' direction of transcription, a transcriptional initiation

regulatory region functional in said host cell, a translational initiation regulatory region functional in said host cell, a DNA sense sequence encoding a plant synthase protein, and a transcriptional and translational termination regulatory region functional in said host cell, wherein said plant synthase protein encoding sequence is under the control of said regulatory regions.

33. The construct of Claim 32 wherein said host cell is a plant cell.

34. The construct of claim 11 wherein said transcriptional initiation region is obtained from a gene preferentially expressed in plant seed tissue.

35. The construct of Claim 21 wherein said plant synthase protein is required for preferential activity toward longer acyl-acyl carrier protein fatty acids.

36. The construct of Claim 21 wherein said plant synthase protein is required for preferential activity toward shorter acyl-acyl carrier protein fatty acids.

37. The construct of Claim 21 wherein said sequence is obtainable from *Ricinus communis* or *Brassica*.

38. A host cell comprising a plant synthase protein encoding sequence construct of any one of Claims 21-37.

39. The cell of claim 38 wherein said cell is a plant cell.

40. The cell of Claim 39 further comprising a second recombinant DNA construct comprising a portion of a plant sequence encoding an enzyme capable of affecting plant lipids.

41. The cell of Claim 40 wherein said enzyme is a desaturase or a thioesterase.

42. The cell of Claim 39 wherein said plant cell is *in vivo*.

43. The cell of Claim 39 wherein said plant cell is a *Brassica* plant cell.

44. A transgenic host cell comprising a plant synthase protein expressed from a recombinant DNA sequence.

45. The cell of Claim 44 wherein said host cell is a plant cell.

46. The cell of Claim 45 wherein said plant synthase protein is required for preferential activity toward longer acyl-acyl carrier protein fatty acids.

47. The cell of Claim 45 wherein said plant synthase protein is required for preferential activity toward shorter acyl-acyl carrier protein fatty acids.

48. A method of producing a plant synthase protein in a host cell or progeny thereof comprising

growing a host cell or progeny thereof comprising a construct of any one of Claims 32-34, under conditions which will permit the production of said plant synthase protein.

49. The method of Claim 48 wherein said host cell is a plant cell and said construct is integrated into the genome of said plant cell.

50. The method of Claim 49 wherein said plant cell is *in vivo*.

51. A host cell comprising a plant synthase protein produced according to Claim 48.

52. A cell of Claim 51 wherein said host cell is a plant host cell and said construct is integrated into the genome of said plant cell.

53. A method of modifying the fatty acid composition in a plant cell comprising:

growing a plant cell having integrated in its genome a DNA construct, said construct comprising in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in said plant cell and a plant synthase protein encoding sequence, under conditions which will permit the transcription of said plant synthase protein encoding sequence.

54. The method of Claim 53 wherein said plant synthase protein encoding sequence is an anti-sense sequence.

55. The method of Claim 54 wherein said plant synthase protein is a synthase factor B.

56. The method of Claim 53 wherein said construct further comprises a translational regulatory region

functional in said plant cell immediately 5' to said plant synthase protein encoding sequence and a transcriptional/ translational termination regulatory region 3' to said sequence and wherein said plant synthase protein encoding sequence is a sense sequence.

57. The method of Claim 53 wherein said plant cell is an oilseed embryo plant cell.

58. A plant cell having a modified free fatty acid composition produced according to the method of any one of Claims 53-57.

59. A plant seed having a modified fatty acid composition as compared to a seed of said plant having a native fatty acid composition, produced according to a method comprising:

growing a plant, having integrated into the genome of embryo cells a recombinant DNA sequence comprising at least a portion of a plant synthase protein encoding sequence under the transcriptional control of regulatory elements functional in seed during lipid accumulation, to produce seed under conditions which will promote the activity of said regulatory elements, and harvesting said seed.

60. The seed of Claim 59 wherein said plant is *Brassica napus*.

61. The seed of Claim 59 wherein said seed is an oilseed.

62. A method of modifying the fatty acid composition of triglycerides produced from an oilseed crop plant comprising:

growing a plant cell having integrated in its genome a DNA construct, said construct comprising in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in said plant cell and a plant synthase protein encoding sequence, under conditions which will permit the transcription of said plant synthase protein encoding sequence.

63. The method of Claim 62 wherein said plant synthase protein encoding sequence is an anti-sense sequence.

64. The method of Claim 62 wherein said construct further comprises a translational regulatory region functional in said plant cell immediately 5' to said plant synthase protein encoding sequence and a transcriptional/ translational termination regulatory region 3' to said sequence and wherein said plant synthase protein encoding sequence is a sense sequence.

65. The method of Claim 62 wherein said plant cell is an oilseed embryo plant cell.

66. A plant cell having a modified fatty acid composition of triglycerides produced according to the method of any one of Claims 62-65.

67. The method of any one of Claims 62 to 65 wherein said crop plant is selected from the group consisting of rapeseed, sunflower, safflower, cotton, cuphea, soybean, peanut, coconut, oil palm and corn.

68. A plant seed oil separated from a seed produced according to any one of Claims 58-60.

69. The oil of Claim 68 comprising a *Brassica* oil.

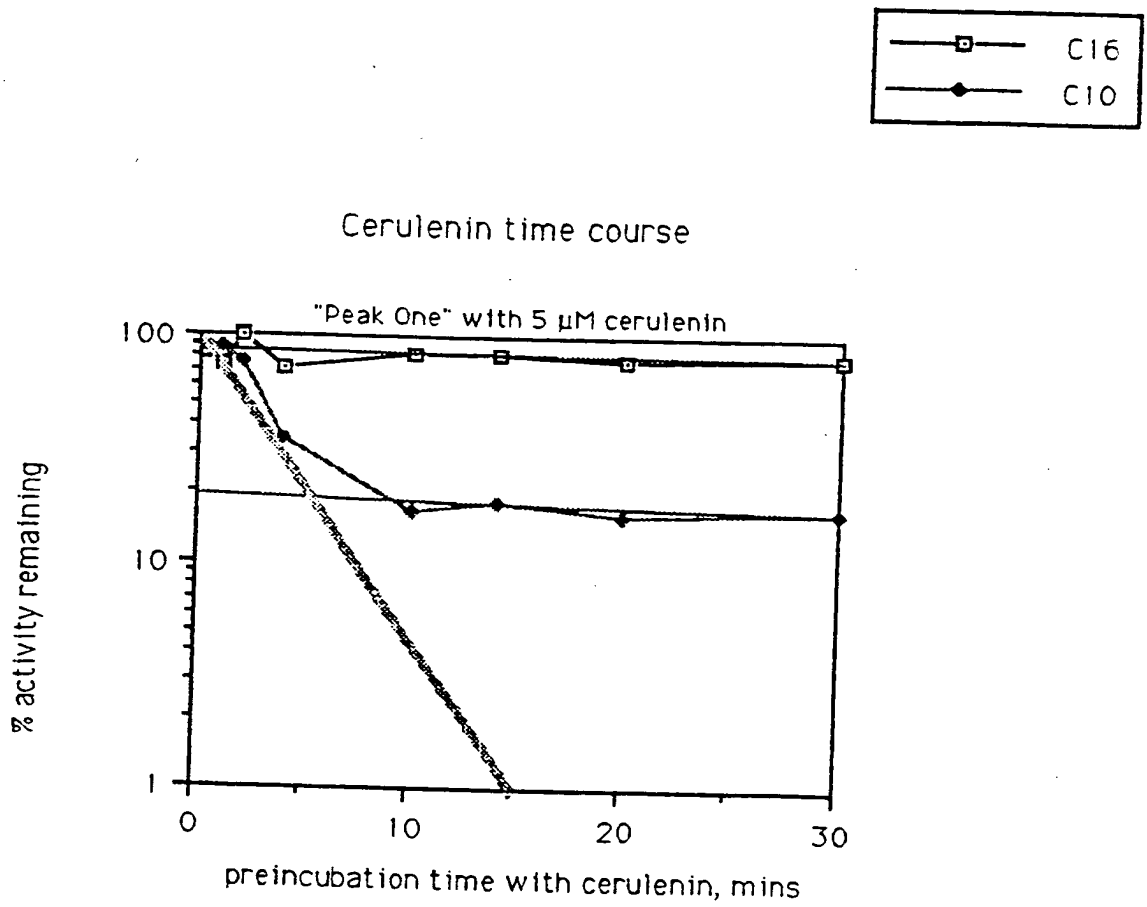
C16
C10
Cerulenin time course
"Peak One" with 5 µM cerulenin
100
10
1
% activity remaining
0
10
20
30
preincubation time with cerulenin, mins

FIG. 1

F1: NTTISAPKKR

F2: VVITGTGLVSVFGNxVDTY

F3: LLAGESTIGLID

F4: GFNSQG$^{Y}_{W}$IDGK

F5: YxIVAGK

F6: ALEHADLGGDK

F7: AGVLVGTGMGGLTVFSDGVQALIxK

F8: ALSQR

F9: NDDPQTASR

F10: DGFVMGEGAGVLVMESL

F11: GAPIIAEYLGGAVNCDAYxMTDP

F12: ADGLGVSSCIER

F13: SLEDAGVSPEEVNYINAHATSTLAGDLAEIN

FIG. 2

KR1: SFSTDGWVAPK

KR2: EEVNYINAxTSTPAGDL

KR3: VFNDAIEALR

KR4: DGFVMGEGAGVLLL

KR7: VVVTGMGVVxPL

KR8: SMIGxLLGAAGAVEAIATIEAI

KR10: GGVxPNINLENPEEGV

KR11: xGVxKEEVNYINAxATxTPAG

KR12: xxPNINLENPEEGV

NT: KHPLMKQRRVVVTGMxV

FIG. 3

Partial Amino Acid Sequences From 50 kD Peptides

KR4

N	D	D	P	Q	T	A
AAT	GAT	GAT	CCN	CAA	ACN	GCN
C	C	C		G		

KR16

E	E	V	N	Y	I	N	A
GAA	GAA	GTN	AAT	TAT	ATT	AAT	GCN
G	G		C	C	C	C	
					A		

Forward Primers

5'GACAAGCTT AAQ GAQ GAQ CCQ CAP ACN GC3' 652-2
5'GACAAGCTT AAQ GAQ GAQ CCP CAP ACN GC3' 652-3

Reverse Primers
(complements)

665-3 3'CTQ CTQ CAN TTP ATP TAP TTP CG CTTAAGCAG

FIG. 4

```
1   GGCTTCTCCCAATTCATCGTTTGGATCGCTACCACTTCCGCCACCACCCCACCACCATGCAAGCCCTGC   69
                                                        METGlnAlaLeuG

          PstI
           |
70  AGTCCCCGTCTCTCCGACCATCCCCTCTAACCCCGCTCCATAAAAATACTCACAATGCAGCAAAACGCC  138
    lnSerProSerLeuArgProSerProLeuThrProLeuHisLysAsnThrHisAsnAlaAlaLysArgP

139 CAACTAAAAAGGTCTCCTTTATCACCGCATCATCAACAAATAACAACACGACGATTTCAGCTCCAAAGC  207
    roThrLysLysValSerPheIleThrAlaSerSerThrAsnAsnAsnThrThrIleSerAlaProLysA

208 GAGAGAAAGACCCCAGAAAAAGGGTAGTCATAACTGGTACGGGTTTGGTATCTGTGTTTGGGAATGATG  276
    rgGluLysAspProArgLysArgValValIleThrGlyThrGlyLeuValSerValPheGlyAsnAspV

277 TCGATACTTACTACGATAAATTGCTTGCTGGAGAAAGTGGGATCGGACTTATTGATAGGTTCGATGCGT  345
    alAspThrTyrTyrAspLysLeuLeuAlaGlyGluSerGlyIleGlyLeuIleAspArgPheAspAlaS
```

FIG. 5A - 1

346 CTAAGTTTCCTACTAGATTTGGTGGACAGATCAGGGGGTTTAATTCACTTGGTTATATTGATGGGAAAA 414
erLysPheProThrArgPheGlyGlyGlnIleArgGlyPheAsnSerLeuGlyTyrIleAspGlyLysA

SphI

415 ATGATAGAAGGCTTGATGATTGTTTGAGGTATTGCATTGTTGCTGGTAAAAAAGCTCTTGAGCATGCTG 483
snAspArgArgLeuAspAspCysLeuArgTyrCysIleValAlaGlyLysLysAlaLeuGluHisAlaA

484 ATCTTGGTGGTGATAAGTTGTCTAAGATTGATAAAGAGCGAGCTGGTGTGCTTGTTGGAACAGGGATGG 552
spLeuGlyGlyAspLysLeuSerLysIleAspLysGluArgAlaGlyValLeuValGlyThrGlyMETG

553 GTGGTCTTACAGTCTTTTCAGATGGTGTTCAGGCCCTAATTGAAAAAGGACACAGGAAAATTACCCCAT 621
lyGlyLeuThrValPheSerAspGlyValGlnAlaLeuIleGluLysGlyHisArgLysIleThrProP

622 TCTTTATTCCTTATGCTATAACAAACATGGGATCTGCCTTGTTAGCTATTGAACTTGGTCTCATGGGTC 690
hePheIleProTyrAlaIleThrAsnMETGlySerAlaLeuLeuAlaIleGluLeuGlyLeuMETGlyP

FIG. 5A - 2

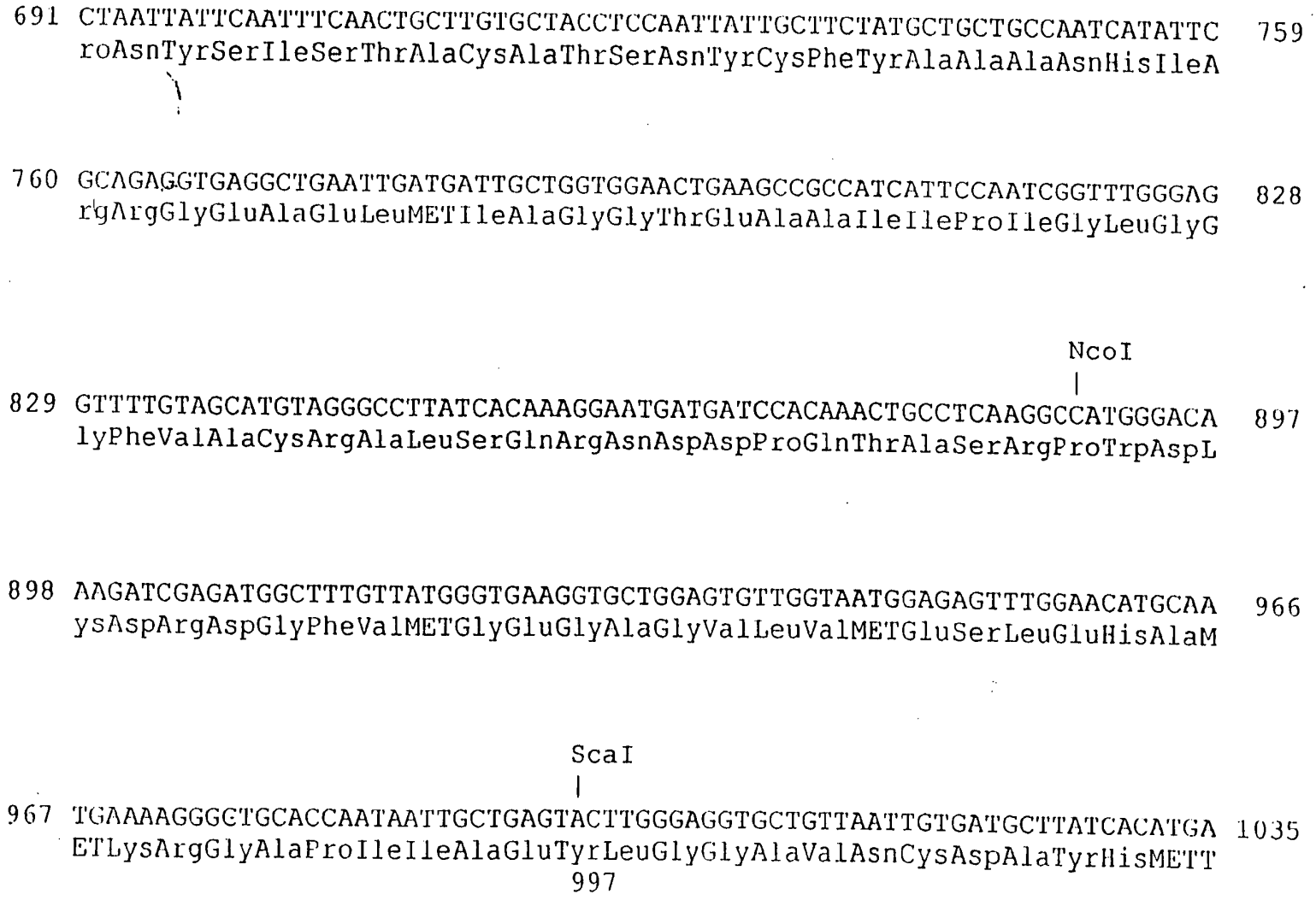

FIG. 5A - 3

```
1036 CTGATCCAAGGGCTGATGGACTTGGGGTCTCTTCCTGCATTGAGAGAAGTCTTGAAGATGCCGGTGTGT 1104
     hrAspProArgAlaAspGlyLeuGlyValSerSerCysIleGluArgSerLeuGluAspAlaGlyValS

                     HpaI
                     |
1105 CACCTGAGGAGGTTAACTATATAAATGCACATGCAACTTCCACTCTTGCTGGTGACCTTNCTGAGATAA 1173
     erProGluGluValAsnTyrIleAsnAlaHisAlaThrSerThrLeuAlaGlyAspLeu   GluIleA
                     1119

1174 ATGCTATTAAAAAAGTATTCAAGAATACGTCTGACATCAAAATCAATGCAACCAAGTCTATGATAGGAC 1242
     snAlaIleLysLysValPheLysAsnThrSerAspIleLysIleAsnAlaThrLysSerMETIleGlyH

1243 ATTGCCTTGGTGCTGCTGGAGGTCTGGAAGCAATTGCCTGTGTGAAGGCCATTACCACAGGATGGTTGC 1311
     isCysLeuGlyAlaAlaGlyGlyLeuGluAlaIleAlaCysValLysAlaIleThrThrGlyTrpLeuH

1312 ATCCTACAATTAATCAATTTAACCCAGAGCCATCAGTTGAATTTGACACTGTTGCCAATAAGAAGCAGC 1380
     isProThrIleAsnGlnPheAsnProGluProSerValGluPheAspThrValAlaAsnLysLysGlnG
```

FIG. 5A - 4

```
1381 AGCACGAAGTGAATGTTGCCATTTCAAATTCCTTTGGATTCGGTGGACACAACTCTGTGGTAGCCTTTT 1449
     lnHisGluValAsnValAlaIleSerAsnSerPheGlyPheGlyGlyHisAsnSerValValAlaPheS

                                                                    NcoI
                                                                     |  |
1450 CTGCATTTAAACCCTGAGAGCATGGTTTTCTTCTGCATTCGGGCCGCGGTCATTTACATTTACCATGGC 1518
     erAlaPheLysPro .

1519 CTGCATTTCTTGTAGGAACCACTGGAGAGTTGCTTGCTTATAGACAGAGTCATCGACATCACTTCCCCC 1587

1588 TTTTAGCTTTTTGAGCTGCTGATAGTAGTCAGTTTCTCATTTCAGTATCAAGTCTATCTTAAGAAGGTC 1656

1657 TTGCTTATTTTTCTTT 1672
```

FIG. 5A - 5

```
  1 GGCTTCTCCCAATTCATCGTTGTTATCGCTACCACTTCCGCCACCACCCCACCACCATGCAAGCCCTGC   69
    LeuLeuProIleHisArgCysTyrArgTyrHisPheArgHisHisProThrThrMETGlnAlaLeuG

 70 AGTCCGCGTCTCTCCGACCATCCCCTCTAACCCCGCTCCATAAAAATACTCACAATGCAGCAAAACGCC  138
    lnSerProSerLeuArgProSerProLeuThrProLeuHisLysAsnThrHisAsnAlaAlaLysArgP

139 CAACTAAAAAGGTCTCCTTTATCACCGCATCATCAACAAATAACAACACGACGATTTCAGCTCCAAAGC  207
    roThrLysLysValSerPheIleThrAlaSerSerThrAsnAsnAsnThrThrIleSerAlaProLysA

208 GAGAGAAAGACCCCAGAAAAAGGGTAGTCATAACTGGTACGGGTTTGGTATCTGTGTTTGGGAATGATG  276
    rgGluLysAspProArgLysArgValValIleThrGlyThrGlyLeuValSerValPheGlyAsnAspV

277 TCGATACTTACTACGATAAATTGCTTGCTGGAGAAAGTGGGATCGGACTTATTGATAGGTTCGATGCGT  345
    alAspThrTyrTyrAspLysLeuLeuAlaGlyGluSerGlyIleGlyLeuIleAspArgPheAspAlaS
```

FIG. 5B - 1

```
346 CTAAGTTTCCTACTAGATTTGGTGGACAGATCAGGGGGTTTAATTCACAAGGTTATATTGATGGGAAAA  414
    erLysPheProThrArgPheGlyGlyGlnIleArgGlyPheAsnSerGlnGlyTyrIleAspGlyLysA

415 ATGATAGAAGGCTTGATGATTGTTTGAGGTATTGCATTGTTGCTGGTAAAAAAGCTCTTGAGCATGCTG  483
    snAspArgArgLeuAspAspCysLeuArgTyrCysIleValAlaGlyLysLysAlaLeuGluHisAlaA

484 ATCTTGGTGGTGATAAGTTGTCTAAGATTGATAAAGAGCGAGCTGGTGTGCTTGTTGGAACAGGGATGG  552
    spLeuGlyGlyAspLysLeuSerLysIleAspLysGluArgAlaGlyValLeuValGlyThrGlyMETG

553 GTGGTCTTACAGTCTTTTCAGATGGTGTTCAGGCCCTAATTGAAAAAGGACACAGGAAAATTACCCCAT  621
    lyGlyLeuThrValPheSerAspGlyValGlnAlaLeuIleGluLysGlyHisArgLysIleThrProP

622 TCTTTATTCCTTATGCTATAACAAACATGGGATCTGCCTTGTTAGCTATTGAACTTGGTCTCATGGGTC  690
    hePheIleProTyrAlaIleThrAsnMETGlySerAlaLeuLeuAlaIleGluLeuGlyLeuMETGlyP

691 CTAATTATTCAATTTCAACTGCTTGTGCTACCTCCAATTATTGCTTCTATGCTGCTGCCAATCATATTC  759
    roAsnTyrSerIleSerThrAlaCysAlaThrSerAsnTyrCysPheTyrAlaAlaAlaAsnHisIleA
```

FIG. 5B - 2

760 GCAGAGGTGAGGCTGAATTGATGATTGCTGGTGGAACTGAAGCCGCCATCATTCCAATCGGTTTGGGAG 828
rgArgGlyGluAlaGluLeuMETIleAlaGlyGlyThrGluAlaAlaIleIleProIleGlyLeuGlyG

829 GTTTTGTAGCATGTAGGGCCTTATCACAAAGGAATGATGATCCACAAACTGCCTCAAGGCCATGGGACA 897
lyPheValAlaCysArgAlaLeuSerGlnArgAsnAspAspProGlnThrAlaSerArgProTrpAspL

898 AAGATCGAGATGGCTTTGTTATGGGTGAAGGTGCTGGAGTGTTGGTAATGGAGAGTTTGGAACATGCAA 966
ysAspArgAspGlyPheValMETGlyGluGlyAlaGlyValLeuValMETGluSerLeuGluHisAlaM

967 TGAAAAGGGGTGCACCAATAATTGCTGAGTACTTGGGAGGTGCTGTTAATTGTGATGCTTATCACATGA 1035
ETLysArgGlyAlaProIleIleAlaGluTyrLeuGlyGlyAlaValAsnCysAspAlaTyrHisMETT

1036 CTGATCCAAGGGCTGATGGACTTGGGGTCTCTTCCTGCATTGAGAGAAGTCTTGAAGATGCCGGTGTGT 1104
hrAspProArgAlaAspGlyLeuGlyValSerSerCysIleGluArgSerLeuGluAspAlaGlyValS

1105 CACCTGAGGAGGTTAACTATATAAATGCACATGCAACTTCCACTCTTGCTGGTGACCTTGCTGAGATAA 1173
erProGluGluValAsnTyrIleAsnAlaHisAlaThrSerThrLeuAlaGlyAspLeuAlaGluIleA

FIG. 5B - 3

```
1174 ATGCTATTAAAAAAGTATTCAAGAATACGTCTGACATCAAAATCAATGCAACCAAGTCTATGATAGGAC 1242
     snAlaIleLysLysValPheLysAsnThrSerAspIleLysIleAsnAlaThrLysSerMETIleGlyH

1243 ATTGCCTTGGTGCTGCTGGAGGTCTGGAAGCAATTGCCTGTGTGAAGGCCATTACCACAGGATGGTTGC 1311
     isCysLeuGlyAlaAlaGlyGlyLeuGluAlaIleAlaCysValLysAlaIleThrThrGlyTrpLeuH

1312 ATCCTACAATTAATCAATTTAACCCAGAGCCATCAGTTGAATTTGACACTGTTGCCAATAAGAAGCAGC 1380
     isProThrIleAsnGlnPheAsnProGluProSerValGluPheAspThrValAlaAsnLysLysGlnG

1381 AGCACGAAGTGAATGTTGCCATTTCAAATTCCTTTGGATTCGGTGGACACAACTCTGTGGTAGCCTTTT 1449
     lnHisGluValAsnValAlaIleSerAsnSerPheGlyPheGlyGlyHisAsnSerValValAlaPheS

1450 CTGCATTTAAACCCTGAGAGCATGGCCTTCTTCTGCATTCGGGCCGCGGTCATTTACATTTACCATGGC 1518
     erAlaPheLysPro

1519 CTGCATTTCTTGTAGGAACCACTGGAGAGTTGCTTGCTTATAGACAGAGTCATCGACATCACTTCCCCC 1587
```

FIG. 5B - 4

```
1588 TTTTAGCTTTTTGAGCTGCTGATAGTAGTCAGTTTCTCATTTCAGTATCAAGTCTATCTTAAGAAGGTC 1656

1657 TTGCTTAATTTTTCTTTTCAAATTACCATTTCATTGTCATTTTCCTTGGAACTTTTAGCTTAAGATCTG 1725

1726 CTGTGATCATGTGGTTTTGATTTCAAATTAATTATGTAGCGGATACGAACAAGCAATCATAAAAAGTCT 1794

1795 TTTTGAATTATGTAATTACGATAACTGTTATTTTCTTTTTCAAAAAAAAAA 1845
```

FIG. 5B - 5

```
          140         150         160         170         180         190
134   AGKKALEHADLGGDKLSKIDKERAGVLVGTGMGGLTVFSDGVQA-LIEKGHRKITPFF
          ||  |           |  |    |  |          |   |       |       |
 76   FLSMEQAIADAGLSPEAYQNNPRVGLIAGSGGGSPRFQVFGADAMRGPRGLKAVGPYV
        80          90         100         110         120         130

          200         210         220         230         240
IPYAITNMGSALLAIELGLMGPNYSISTACATSNYCFYAAANHIRRGEAELMIAGGTE
   |      ||  ||       | |||||  |||||   |    |    |   |        |||  |
VTKAMASGVSACLATPFKIHGVNYSISSACATSAHCIGNAVEQIQLGKQDIVFAGGGE
     140         150         160         170         180         190

250         260         270         280         290         300
AAIIPIGLGGFVACRALS-QRNDDPQTASRPWDKDRDGFVMGEGAGVLVMESLEHAMK
            |  |   |||    ||  |   |||   |   |||||    |  |   |  |  ||||
-ELCWEMACEFDAMGALSTKYNDTPEKASRTYDAHRDGFVIAGGGGMVVVEELEHALA
          200         210         220         230         240
```

FIG. 6A - 1

```
   310         320         330         340         350         360
 RGAPIIAEYLGGAVNCDAYHMTDPRADGLGVSSCIERSLEDAGVSPEEVNYINAHATS
 ||| | || |       |    | | |              |          | | | ||
 RGAHIYAEIVGYGATSDGADMVAPSGEGAVRCMKMAMHGVD-----TPIDYLNSHGTS
250         260         270         280             290         300

     370         380         390
 TLAGDLXEINAIKKVFKNTSDIKINATKSMIGH    396
 |  || | |  ||  ||   |   | ||| | ||
 TPVGDVKELAAIREVFGDKS-PAISATKAMTGH    333
       310         320         330
```

FIG. 6A - 2

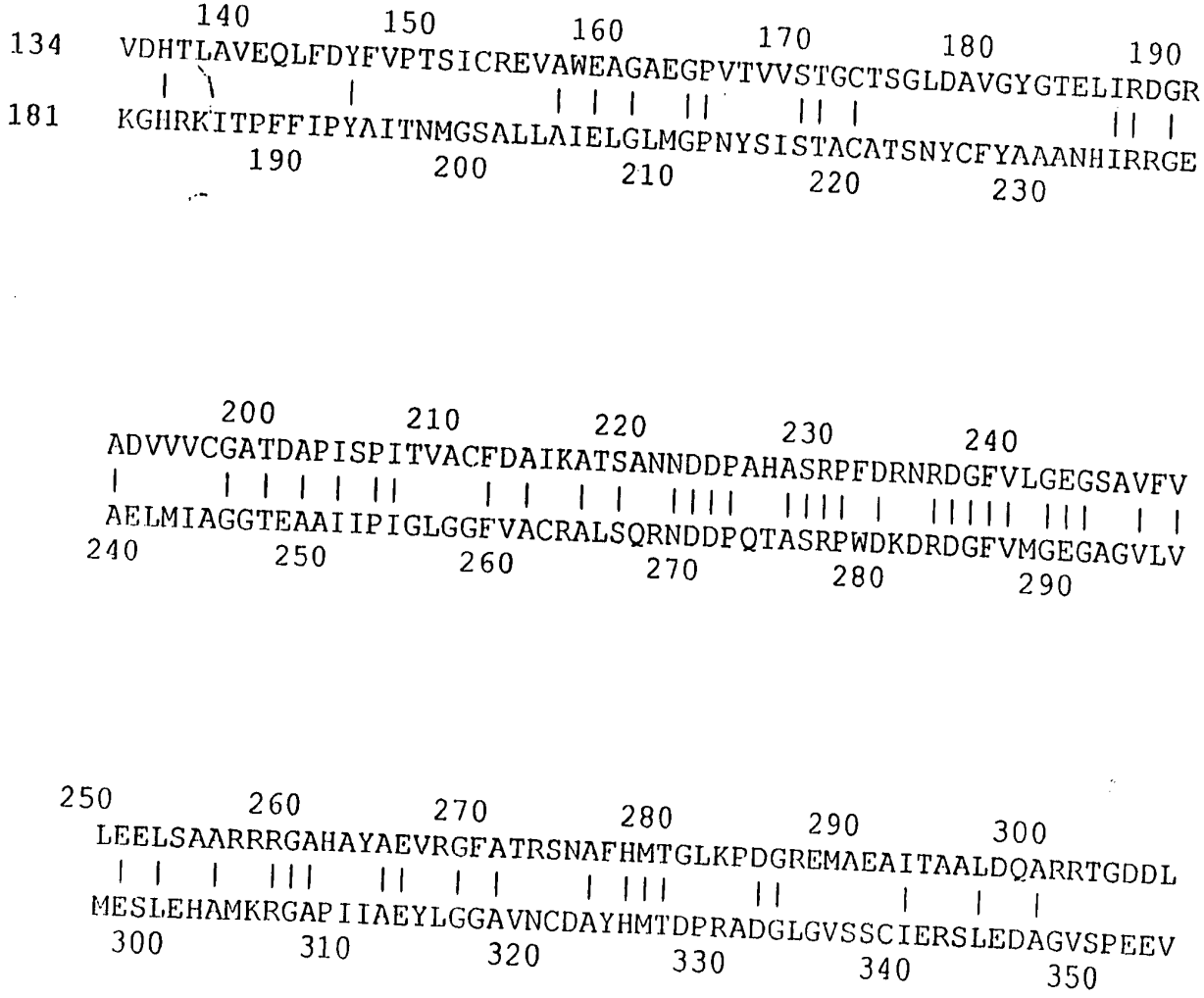
140 150 160 170 180 190
134 VDHTLAVEQLFDYFVPTSICREVAWEAGAEGPVTVVSTGCTSGLDAVGYGTELIRDGR
181 KGHRKITPFFIPYAITNMGSALLAIELGLMGPNYSISTACATSNYCFYAAANHIRRGE
190 200 210 220 230
200 210 220 230 240
ADVVVCGATDAPISPITVACFDAIKATSANNDDPAHASRPFDRNRDGFVLGEGSAVFV
AELMIAGGTEAAIIPIGLGGFVACRALSQRNDDPQTASRPWDKDRDGFVMGEGAGVLV
240 250 260 270 280 290
250 260 270 280 290 300
LEELSAARRRGAHAYAEVRGFATRSNAFHMTGLKPDGREMAEAITAALDQARRTGDDL
MESLEHAMKRGAPIIAEYLGGAVNCDAYHMTDPRADGLGVSSCIERSLEDAGVSPEEV
300 310 320 330 340 350

FIG. 6B - 1

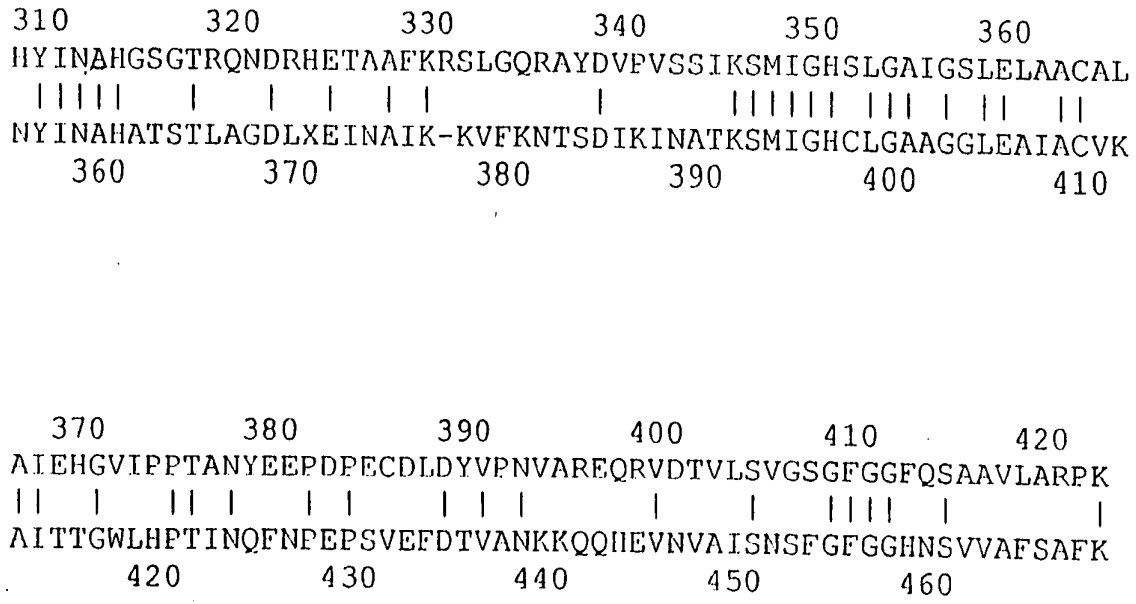
310 320 330 340 350 360
HYINAHGSGTRQNDRHETAAFKRSLGQRAYDVPVSSIKSMIGHSLGAIGSLELAACAL
NYINAHATSTLAGDLXEINAIK-KVFKNTSDIKINATKSMIGHCLGAAGGLEAIACVK
360 370 380 390 400 410
370 380 390 400 410 420
AIEHGVIPPTANYEEPDPECDLDYVPNVAREQRVDTVLSVGSGFGGFQSAAVLARPK 422
AITTGWLHPTINQFNPEPSVEFDTVANKKQQHEVNVAISNSFGFGGHNSVVAFSAFK 468
420 430 440 450 460

FIG. 6B - 2

```
   1 ATGATTACCTGAAAATAAGTATAATTTGTATTGAAATTATAAAGTGACATTTTTTGTGTAACAAATATT   69

  70 TTGTGTAACAAGAATTAAAAAAAAAAACAGAAAATACTCAGCTTTTTTAATAATAAAAAAAATTAATTG  138

 139 AGTTAGAAAATTGTTGTACCAATAACAAAAGATTTATATGGAATTATAAAATCAACACACCAATAACAC  207

 208 AAGACTTTTTAAAAATTTAAGAATAATATAAGCAATAACAATAGAATCTTCAAATTCTTCAAATCCTTA  276

 277 AAAATCAATCTCCCACTATTAATCCCCCTTAGTTTTAGTTGGTAATGGCAACGTTTGTTGACTACCGTA  345

 346 TTGTAACTTTTGTCAAATTGTCATAAATACGTGTCAAACTCTGGTAAAAAATTAGTCTGCTACATCTGT  414
```

FIG. 7 - 1

```
415 CTTTTATTTATAAAACACAGCTGTTAATCAGAATTTGGTTTATTAAATCAACAACCTGCACGAAACTTG  483

484 TGTGAGCATATTTTGTCTGTTTCTGGTTCATGACCTTCTTCCGCATGATGGCCAAGTGTAATGGCCACT  552

                                                          BglII
                                                            |
553 TGCAAGAGCGTTTCTTCAACGAGATAAGTCGAACAAATATTTGTCCGTTACGACCACATATAANATCTC  621
                                                          616

622 CCCATCTCTATATATAATACCAGCATTCACCATCATGAATACCTCAAATCCCAATCTCACAAATACTTC  690

691 AATAAAAAGACCAAAAAAAAATTAAAGCAAAGAAAAGCCTTCTTGTGCACAAAAAAAAAAGAAGCCTTCT  759
```

FIG. 7 - 2

```
760 AGGTTTTCACGACATGAAGTTCACTACTCTAATGGTCATCACATTGGTGATAATCGCCATCTCGTCTCC  828
                METLysPheThrThrLeuMETValIleThrLeuValIleIleAlaIleSerSerPr

829 TGTTCCAATTAGAGCAACCACGGTTGAAAGTTTCGGAGAAGTGGCACAATCGTGTGTTGTGACAGAACT  897
    oValProIleArgAlaThrThrValGluSerPheGlyGluValAlaGlnSerCysValValThrGluLe

898 CGCCCCATGCTTACCAGCAATGACCACGGCAGGAGACCCGACTACAGAATGCTGCGACAAACTGGTAGA  966
    uAlaProCysLeuProAlaMETThrThrAlaGlyAspProThrThrGluCysCysAspLysLeuValGl

967 GCAGAAACCATGTCTTTGTGGTTATATTCGAAACCCAGCCTATAGTATGTATGTTACTTCTCCAAACGG 1035
    uGlnLysProCysLeuCysGlyTyrIleArgAsnProAlaTyrSerMETTyrValThrSerProAsnGl

1036 TCGCAAAGTCTTAGATTTTTGTAAGGTTCCTTTTCCTAGTTGTTAAATCTCTCAAGACATTGCTAAGAA 1104
     yArgLysValLeuAspPheCysLysValProPheProSerCys .
```

FIG. 7 - 3

```
                                        BglII                                                   HindII
                                          |                                                       |
1105 AAATATTATTAAAAATAAAAGAATCAAACTAGATCTGATGTAACAATGAATCATCATGTTATGGTTGAA 1173
                                        1136                                                    1173

1174 GCTTATATAGCTGAAGTGTTTTGATTTTATATATGTGTGTGTGTGTGTCCTGCTCAATTTTTGAAACAC 1242

1243 ACACGTTTCTCCTGATTTGGATTTAAATTATATTTTGAGTTAAAAAAAAGAAAAAGATGGAATGCTATT 1311

                                              EcoRV
                                                |
1312 TATACAAGTTGATGAAAAAGTGGAAGTACAATTTAGATATCTCCWWCACTTAAAGAATGAAACAATAAT 1380
                                              1350
```

FIG. 7 - 4

SalI

1381 AGACTTCGAAACAAATGAAAAATACATAAATTGTCGACAATCAACGTCGATCGACGAGTTTATTATTAA 1449

1414

1450 AAATTTGTGTGAAGGACTAGCAGTTCAACCAAATGATATTGAACATATACATCAACAAATATGATAATC 1518

1519 ATAAAAGAGAGAATGGGGGGGGGGTGTCGTTTACCAGAAACCTCTTTTTCTCAGCTCGCTAAAACCCTA 1587

1588 CCACTAGAGACCTAGCTCTGACCGTCGGCTCATCGGTGCCGGAGGTGTAACCTTTCTTTCCCATGACCC 1656

1657 GAAACCTCTCTTTCCCAACTCACGAAAACCCTACAATCAAAAACCTAGCTCCGACCATCGGCTCATCGG 1725

FIG. 7 - 5

ClaI
|
1726 TGCCGAAGGTGTAACCTTTCNCTCCCATCATAGTTTCTCGTAAATGAAAGCTAATTGGGCAATCGATTT 1794
1789

1795 TTTAATGTTTAAACCATGCCAAGCCATTTCTTATAGGACAATTGTCAATAATAGCATCTTTTGAGTTTT 1863

1864 GTCTCAAAAGTGACACTAGAAGAAAAAAGTCACAAAAATGACATTCATTAAAAAGTAAAATATCCCTAA 1932

1933 TACCTTTGGTTTAAATTAAATAAGTAAACAAAAATAAATAAAAACAAATAAAATAAAAATAAAAAATGA 2001

2002 AAAAAAGAAATTTTTTTATAGTTTCAGATTATATGTTTTCAGATTCGAAATTTTTTAAA 2060

FIG. 7 - 6

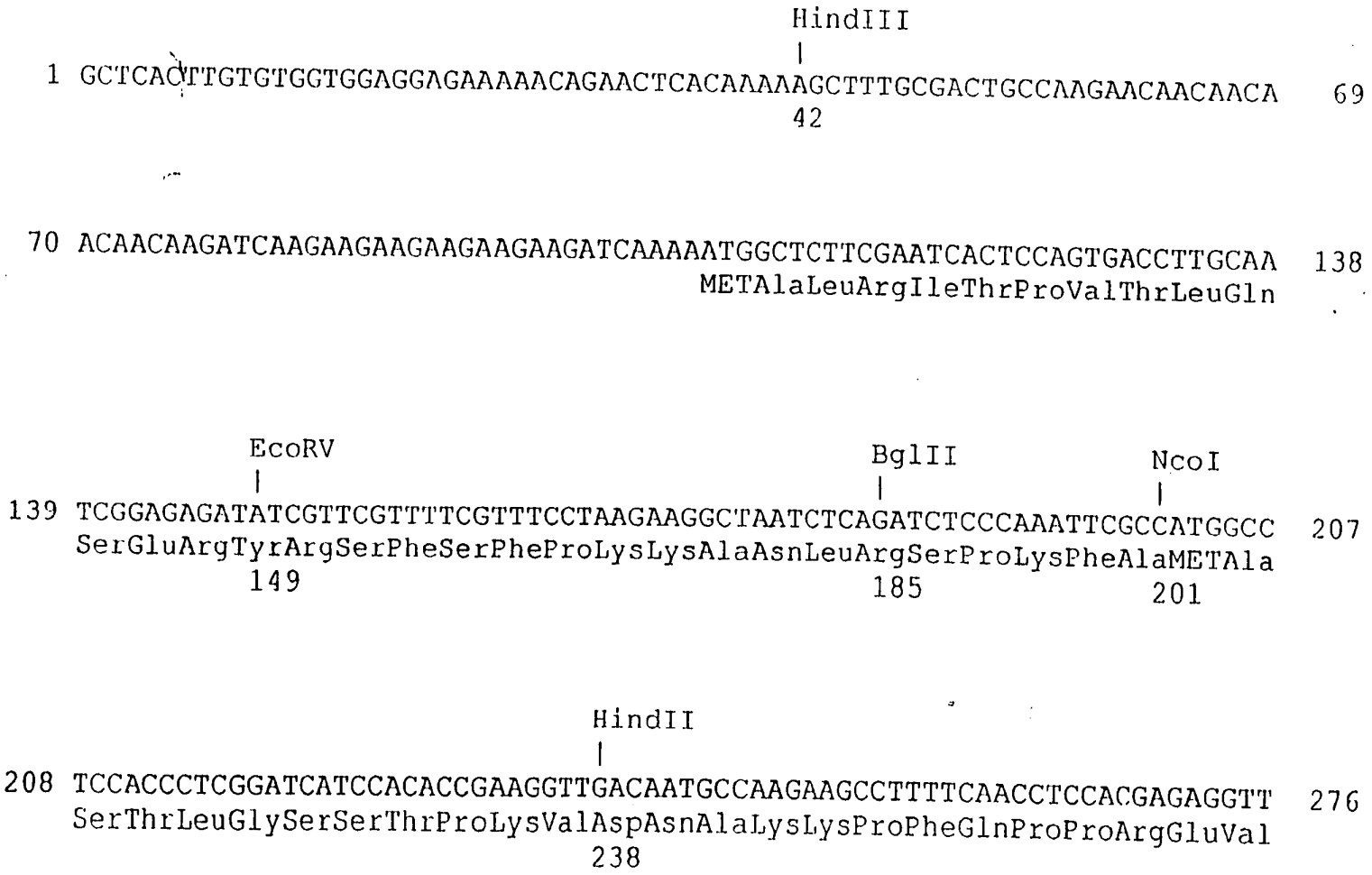

FIG. 8 - 1

```
277 CATGTTCAGGTGACGCACTCCATGCCACCACAGAAGATAGAGATTTTCAAATCCATCGAGGGTTGGGCT 345
    HisValGlnValThrHisSerMETProProGlnLysIleGluIlePheLysSerIleGluGlyTrpAla

346 GAGCAGAACATATTGGTTCACCTAAAGCCAGTGGAGAAATGTTGGCAAGCACAGGATTTCTTGCCGGAC 414
    GluGlnAsnIleLeuValHisLeuLysProValGluLysCysTrpGlnAlaGlnAspPheLeuProAsp

415 CCTGCATCTGAAGGATTTGATGAACAAGTCAAGGAACTAAGGGCAAGAGCAAAGGAGATTCCTGATGAT 483
    ProAlaSerGluGlyPheAspGluGlnValLysGluLeuArgAlaArgAlaLysGluIleProAspAsp

484 TACTTTGTTGTTTTGGTTGGAGATATGATTACAGAGGAAGCCCTACCTACTTACCAAACAATGCTTAAT 552
    TyrPheValValLeuValGlyAspMETIleThrGluGluAlaLeuProThrTyrGlnThrMETLeuAsn

553 ACCCTAGATGGTGTACGTGATGAGACTGGGGCTAGCCTTACGCCTTGGGCTGTCTGGACTAGGGCTTGG 621
    ThrLeuAspGlyValArgAspGluThrGlyAlaSerLeuThrProTrpAlaValTrpThrArgAlaTrp
```

FIG. 8 - 2

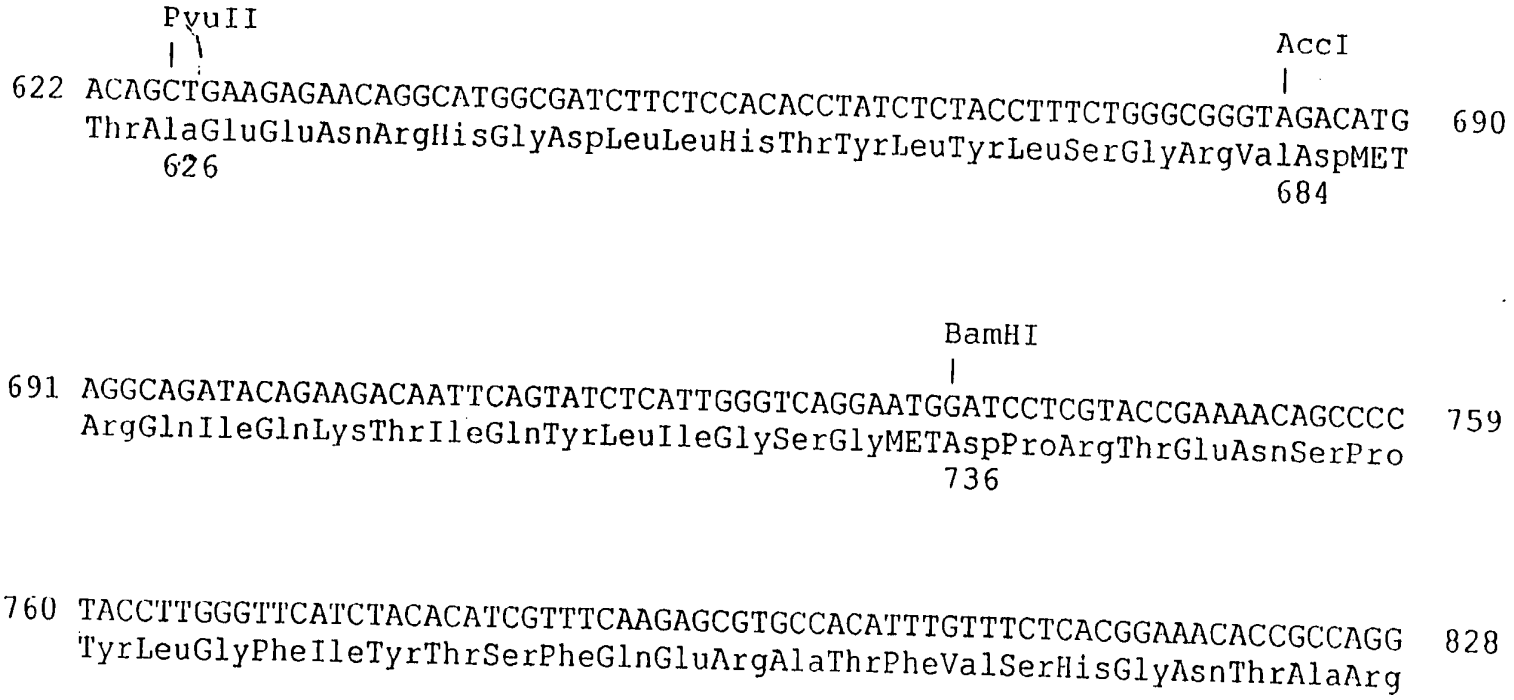

FIG. 8 - 3

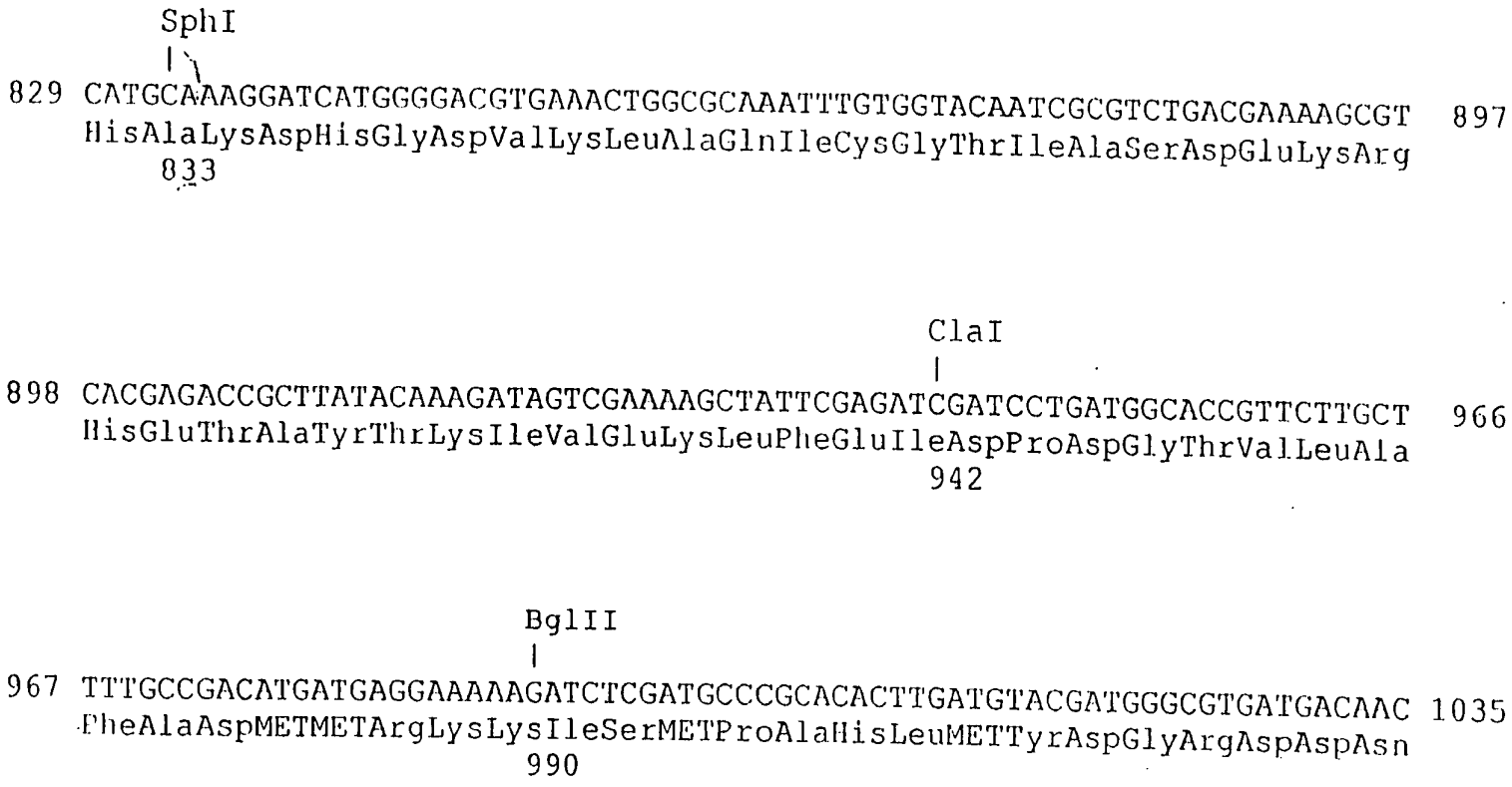
SphI
829 CATGCAAAGGATCATGGGGACGTGAAACTGGCGCAAATTTGTGGTACAATCGCGTCTGACGAAAAGCGT 897
HisAlaLysAspHisGlyAspValLysLeuAlaGlnIleCysGlyThrIleAlaSerAspGluLysArg
833
ClaI
898 CACGAGACCGCTTATACAAAGATAGTCGAAAAGCTATTCGAGATCGATCCTGATGGCACCGTTCTTGCT 966
HisGluThrAlaTyrThrLysIleValGluLysLeuPheGluIleAspProAspGlyThrValLeuAla
942
BglII
967 TTTGCCGACATGATGAGGAAAAAGATCTCGATGCCCGCACACTTGATGTACGATGGGCGTGATGACAAC 1035
PheAlaAspMETMETArgLysLysIleSerMETProAlaHisLeuMETTyrAspGlyArgAspAspAsn
990

FIG. 8 - 4

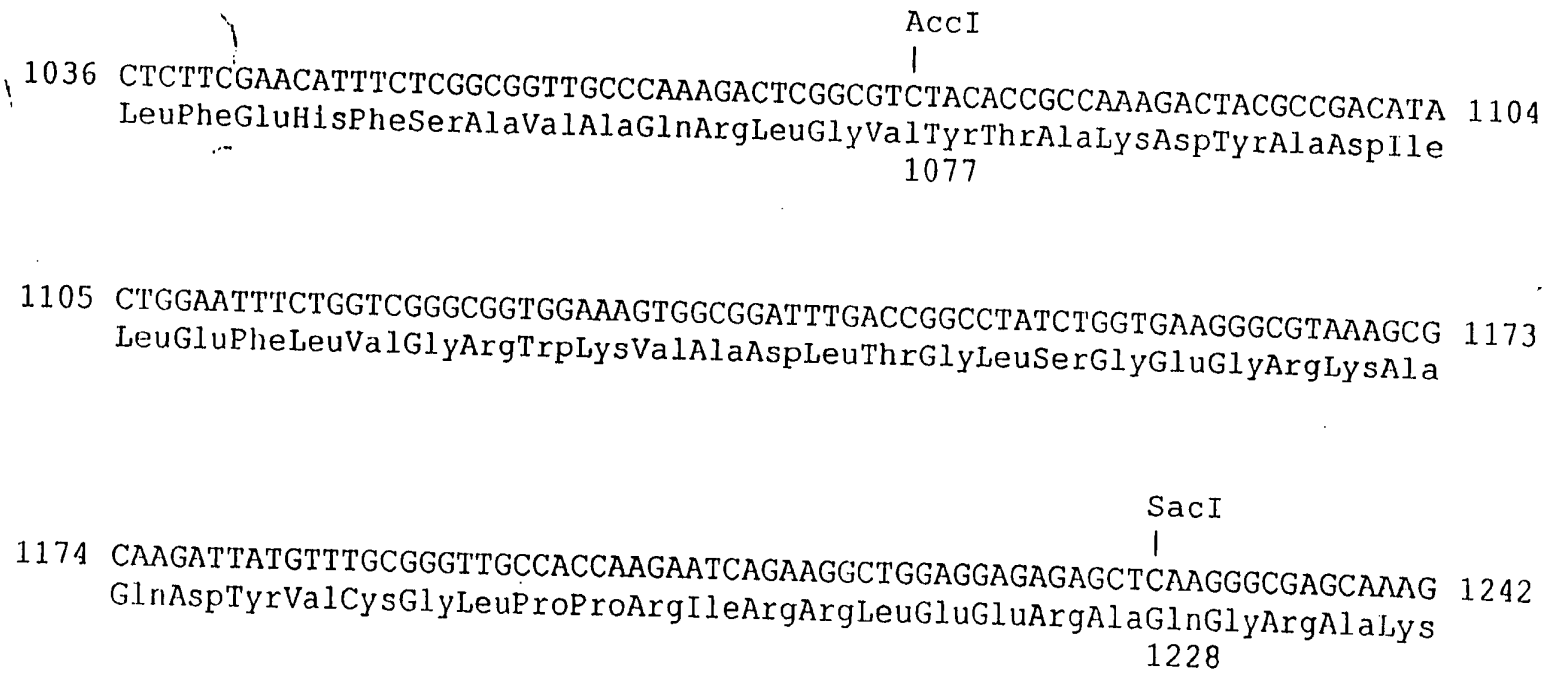

FIG. 8 - 5

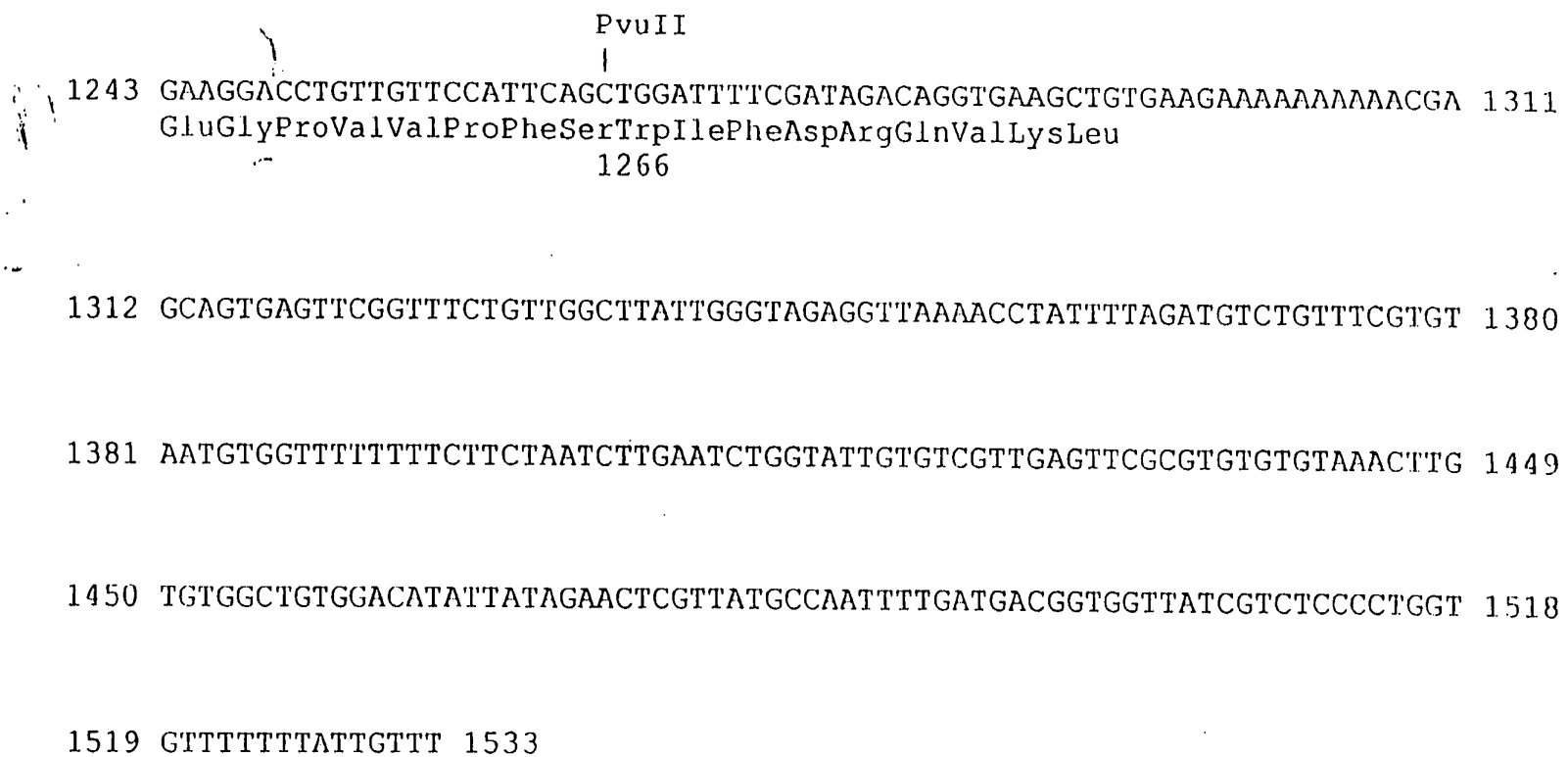
PvuII
1243 GAAGGACCTGTTGTTCCATTCAGCTGGATTTTCGATAGACAGGTGAAGCTGTGAAGAAAAAAAAAAACGA 1311
GluGlyProValValProPheSerTrpIlePheAspArgGlnValLysLeu
1266
1312 GCAGTGAGTTCGGTTTCTGTTGGCTTATTGGGTAGAGGTTAAAACCTATTTTAGATGTCTGTTTCGTGT 1380
1381 AATGTGGTTTTTTTTCTTCTAATCTTGAATCTGGTATTGTGTCGTTGAGTTCGCGTGTGTGTAAACTTG 1449
1450 TGTGGCTGTGGACATATTATAGAACTCGTTATGCCAATTTTGATGACGGTGGTTATCGTCTCCCCTGGT 1518
1519 GTTTTTTTATTGTTT 1533

FIG. 8 - 6

```
 1 TGAGAGATAGTGTGAGAGCATTAGCCTTAGAGAGAGAGAGAGAGAGCTTGTGTCTGAAAGAATCCACAA  69

                HindIII
                |
70 ATGGCATTGAAGCTTAACCCTTTGGCATCTCAGCCTTACAACTTCCCT 117
   METAlaLeuLysLeuAsnProLeuAlaSerGlnProTyrAsnPhePro
```

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FIG. 9A

```
                                        PstI
                                         |
  1 ACTTCATGGGCTATTTGGACAAGAGCTTGGACTGCAGAAGAGAACCGACACGGTGATCTTCTCAATAAG  69
    ThrSerTrpAlaIleTrpThrArgAlaTrpThrAlaGluGluAsnArgHisGlyAspLeuLeuAsnLys

 70 TATCTTTACTTGTCTGGACGTGTTGACATGAGGCAGATTGAAAAGACCATTCAGTACTTGATTGGTTCT 138
    TyrLeuTyrLeuSerGlyArgValAspMETArgGlnIleGluLysThrIleGlnTyrLeuIleGlySer

          BamHI
           |
139 GGAATGGATCCTAGAACAGAGAACAATCCTTACCTCGG 176
    GlyMETAspProArgThrGluAsnAsnProTyrLeuAla
```

FIG. 9B

```
1   CCCCGTGGCGGCGTGCATGTCGGTCACGTGCTCAAAGGAGAACAGACACGCGTTCTTCTCTTCATCGAC   69
     ProValAlaAlaCysMETSerValThrCysSerLysGluAsnArgHisAlaPheSerSerSerTh

70  ACCGGGCACCACCAGCAGTCACAGTCGTACAAGAAGGAGGCCTAAATATAATAGTATCAGCACCCCTGC  138
    rProGlyThrThrSerSerHisSerArgThrArgArgArgProLysTyrAsnSerIleSerThrProAl

139 CTCTCAATCTTTCTTTAATTCTTTATCATCTTCTGGATCGAGTTTTCAACAATTAATGTCTTCTTGCTT  207
    aSerGlnSerPhePheAsnSerLeuSerSerSerGlySerSerPheGlnGlnLeuMETSerSerCysLe

208 GGCCTTCGAGCCTTGTAGTCATTACTACAGCTCTAATGGCCTCTTTCCTAACACTCCTCTTCTTCCTAA  276
    uAlaPheGluProCysSerHisTyrTyrSerSerAsnGlyLeuPheProAsnThrProLeuLeuProLy

277 GCGCCATCCTAGACTTCATCATCGCCTTCCTCGTTCTGGGGAAGCAATGGCAGTGGCTGTGCAACCTGA  345
    sArgHisProArgLeuHisHisArgLeuProArgSerGlyGluAlaMETAlaValAlaValGlnProGl

346 AAAGGAGGTTGCAACAAATAAGAAACCTCTTATGAAGCAAAGGAGAGTAGTTGTTACTGGGATGGGTGT  414
    uLysGluValAlaThrAsnLysLysProLeuMETLysGlnArgArgValValValThrGlyMETGlyVa
```

FIG. 10 - 1

```
415 TGTTTCACCCCTTGGTCATGATATAGACGTCTATTACAATAATCTTCTTGACGGTTCTAGTGGTATTAG  483
    lValSerProLeuGlyHisAspIleAspValTyrTyrAsnAsnLeuLeuAspGlySerSerGlyIleSe

484 TCAGATTGATTCCTTTGACTGTGCCCAATTTCCTACGAGGATTGCTGGAGAGATCAAGTCTTTCTCAAC  552
    rGlnIleAspSerPheAspCysAlaGlnPheProThrArgIleAlaGlyGluIleLysSerPheSerTh

553 TGATGGATGGGTTGCACCAAAACTTTCCAAGAGAATGGATAAATTCATGCTTTACATGCTTACTGCTGG  621
    rAspGlyTrpValAlaProLysLeuSerLysArgMETAspLysPheMETLeuTyrMETLeuThrAlaGl

622 CAAAAAAGCCTTGGCAGATGGTGGTATTACAGAGGATATGATGGATGAATTGGATAAAGCTAGATGTGG  690
    yLysLysAlaLeuAlaAspGlyGlyIleThrGluAspMETMETAspGluLeuAspLysAlaArgCysGl

691 AGTTTTAATTGGTTCTGCAATGGGTGGCATGAAGGTTTTCAATGATGCAATTGAAGCATTAAGGATCTC  759
    yValLeuIleGlySerAlaMETGlyGlyMETLysValPheAsnAspAlaIleGluAlaLeuArgIleSe
```

FIG. 10 - 2

```
760 GTATAGGAAGATGAATCCTTTCTGCGTACCTTTTGCGACTACAAATATGGGCTCTGCCATGCTTGCAAT  828
    rTyrArgLysMETAsnProPheCysValProPheAlaThrThrAsnMETGlySerAlaMETLeuAlaME

829 GGACCTTGGTTGGATGGGGCCAAACTATTCAATATCTACTGCTTGTGCTACTAGCAATTTTTGTATATT  897
    TAspLeuGlyTrpMETGlyProAsnTyrSerIleSerThrAlaCysAlaThrSerAsnPheCysIleLe

898 GAATGCCGCAAACCACATCATTAGAGGCGAAGCTGATATTATGCTTTGTGGTGGCTCAGATGCAGCAAT  966
    uAsnAlaAlaAsnHisIleIleArgGlyGluAlaAspIleMETLeuCysGlyGlySerAspAlaAlaIl

967 TATACCTATTGGCTTGGGAGGTTTTGTGGCATGCAGAGCGCTCTCACAGAGGAATGATGATCCTACAAA 1035
    eIleProIleGlyLeuGlyGlyPheValAlaCysArgAlaLeuSerGlnArgAsnAspAspProThrLy

1036 AGCTTCACGACCTTGGGATATGAATCGGGATGGATTTGTGATGGGGGAAGGAGCTGGTGTTCTTCTTTT 1104
     sAlaSerArgProTrpAspMETAsnArgAspGlyPheValMETGlyGluGlyAlaGlyValLeuLeuLe
```

FIG. 10 - 3

```
1105 AGAAGAACTAGAACATGCTAAGAAAAGAGGTGCAAATATTTATGCGGAATTTCTTGGAGGAAGCTTTAC 1173
     uGluGluLeuGluHisAlaLysLysArgGlyAlaAsnIleTyrAlaGluPheLeuGlyGlySerPheTh

1174 ATGTGATGCTTATCACATGACTGAACCGCGTCCAGATGGAGTTGGTGTCATTCTCTGTATAGAAAAGGC 1242
     rCysAspAlaTyrHisMETThrGluProArgProAspGlyValGlyValIleLeuCysIleGluLysAl

1243 ATTAGCGCGATCTGGTGTATCCAAGGAGGAAGTAAACTACATAAATGCACATGCTACGTCTACCCCAGC 1311
     aLeuAlaArgSerGlyValSerLysGluGluValAsnTyrIleAsnAlaHisAlaThrSerThrProAl

1312 TGGAGACCTTAAAGAATATGAAGCTCTTATGCGCTGTTTCAGCCAAAATCCTGATTTGAGAGTGAACTC 1380
     aGlyAspLeuLysGluTyrGluAlaLeuMETArgCysPheSerGlnAsnProAspLeuArgValAsnSe

1381 TACGAAGTCTATGATTGGCCATTTACTAGGAGCAGCTGGTGCTGTGGAAGCTATAGCAACAATACAGGC 1449
     rThrLysSerMETIleGlyHisLeuLeuGlyAlaAlaGlyAlaValGluAlaIleAlaThrIleGlnAl
```

FIG. 10 - 4

```
1450 GATACGGACAGGATGGGTTCATCCAAACATCAACCTGGAAAACCCAGAAGAAGGCGTGGACACAAAGGT 1518
     aIleArgThrGlyTrpValHisProAsnIleAsnLeuGluAsnProGluGluGlyValAspThrLysVa

1519 GCTGGTTGGCCCAAAGAAGGAGAGATTGGACATTAAGGTTGCTCTGTCCAACTCTTTTGGGTTCGGTGG 1587
     lLeuValGlyProLysLysGluArgLeuAspIleLysValAlaLeuSerAsnSerPheGlyPheGlyGl

1588 GCACAACTCATCGATCATTTTTGCTCCGTACAAGTGAAATAAGGGGTACTTCAACTTTGGTGTATTAAC 1656
     yHisAsnSerSerIleIlePheAlaProTyrLys

1657 GTGAAAGATGATCTAAAATGGAACAAGATTAGATAACTCTATGGGTAGGGAAAGGAGAATATGCCGAGT 1725

1726 TCACAGAGAGGAAACTTCCCGTGAAGATTCCTGTGCCTTCTACCATTTTCAGTATTCTCTCCGCATCAT 1794

1795 TGTGGCTTGATCCATGTTGATCCATCGAATACCAGTAACAGTGGCCTTATTTAATTTTTGTTCCATGTA 1863
```

FIG. 10 - 5

1864 TAAGCAGACGGCTGATCGTTGCTTTAACAGTCAATTGTAATGAATTTTTGAGCTGGACAGTTGGCTAGG 1932

1933 TTACACTAATGTAATGGTGGTTTTATGAGCAAAAAAA 1969

FIG. 10 - 6

```
1   ATGCGAGACAGCCCACGAGAAGACGCTCATTCATCTCCGCGTCGTCCTCCGCCGTCTCCGCCCCCAAAC   69
      AlaArgGlnProThrArgArgArgSerPheIleSerAlaSerSerSerAlaValSerAlaProLysA

70  GCGAAACAGACCCGAAGAAACGGGTCGTAATCACCGGAATGGGCCTCGTCTCCGTCTTCGGAAACGACG  138
    rgGluThrAspProLysLysArgValValIleThrGlyMETGlyLeuValSerValPheGlyAsnAspV

139 TCGACGCTTACTACGAGAAGCTGCTCTCCGGCGAGAGTGGAATCAGCTTGATTGATCGGTTCGACGCCT  207
    alAspAlaTyrTyrGluLysLeuLeuSerGlyGluSerGlyIleSerLeuIleAspArgPheAspAlaS

208 CCAAGTTCCCGACCCGATTCGGTGGACAGATCCGTGGGTTCAGCTCAGAGGGTTACATCGATGGGAAGA  276
    erLysPheProThrArgPheGlyGlyGlnIleArgGlyPheSerSerGluGlyTyrIleAspGlyLysA

277 ATGAGCGGAGGCTTGATGATTGCTTGAAGTACTGCATTGTCGCTGGGAAGAAGGCTCTTGAAAGTGCGA  345
    snGluArgArgLeuAspAspCysLeuLysTyrCysIleValAlaGlyLysLysAlaLeuGluSerAlaA

346 ATCTTGGTGGTGATAAGCTTAACACGATTGATAAGCAGAAAGCTGGAGTACTAGTTGGGACTGGTATGG  414
    snLeuGlyGlyAspLysLeuAsnThrIleAspLysGlnLysAlaGlyValLeuValGlyThrGlyMETG
```

FIG. 11A - 1

```
415 GTGGCTTGACTGTGTTTTCAGACGGTGTTCAAGCTCTTATTGAGAAAGGTCACAGGAGGATTTCTCCTT  483
    lyGlyLeuThrValPheSerAspGlyValGlnAlaLeuIleGluLysGlyHisArgArgIleSerProP

484 TCTTTATTCCTTATGCTATTACAAACATGGGTTCTGCTTTGTTGGCGATTGATCTTGGTCTTATGGGTC  552
    hePheIleProTyrAlaIleThrAsnMETGlySerAlaLeuLeuAlaIleAspLeuGlyLeuMETGlyP

553 CTAACTACTCGATCTCGACGGCTTGTGCCACTTCTAACTACTGCTTTTACGCTGCTGCGAATCACATTC  621
    roAsnTyrSerIleSerThrAlaCysAlaThrSerAsnTyrCysPheTyrAlaAlaAlaAsnHisIleA

622 GACGTGGTGAAGCTGATATGATGATAGCTGGTGGAACCGAGGCTGCTATTATTCCTATTGGTTTGGGAG  690
    rgArgGlyGluAlaAspMETMETIleAlaGlyGlyThrGluAlaAlaIleIleProIleGlyLeuGlyG

691 GTTTTGTTGCTTGTAGGGCGCTTTCACAGAGAAATGATGATCCTCAGACGGCTTCAAGGCCGTGGGATA  759
    lyPheValAlaCysArgAlaLeuSerGlnArgAsnAspAspProGlnThrAlaSerArgProTrpAspL

760 AACAGAGAGATGGGTTTGTCATGGGTGAAGGAGCTGGTGTTCTGGTGATGGAAAGCTTGGAACATGCGA  828
    ysGlnArgAspGlyPheValMETGlyGluGlyAlaGlyValLeuValMETGluSerLeuGluHisAlaM
```

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FIG. 11A - 2

```
829 TGAAACGTGGTGCTCCAATTGTAGCAGAGTATCTTGGAGGCGCTGTTAACTGCGATGCTCATCATATGA  897
    ETLysArgGlyAlaProIleValAlaGluTyrLeuGlyGlyAlaValAsnCysAspAlaHisHisMETT

898 CTGATCCAAGAGCTGATGGGCTTGGTGTGTCTTCATGCATTGAGAGCTGCCTTGAAGATGCTGGTGTAT  966
    hrAspProArgAlaAspGlyLeuGlyValSerSerCysIleGluSerCysLeuGluAspAlaGlyValS

967 CACCTGAGGAGGTAAATTACATCAATGCACATGCAACTTCCACACTGGCTGGTGATCTTGCTGAGATTA 1035
    erProGluGluValAsnTyrIleAsnAlaHisAlaThrSerThrLeuAlaGlyAspLeuAlaGluIleA

1036 ATGCCATTAAAAAGGTATTCAAAAGCACTTCAGGGATCAAAATCAATGCCACCAAGTCTATGATAGGTC 1104
     snAlaIleLysLysValPheLysSerThrSerGlyIleLysIleAsnAlaThrLysSerMETIleGlyH

1105 ACTGCCTCGGTGCAGCTGGAGGTCTTGAAGCCATTGCCACCGTGAAGGCTATCAACACGGGATGGCTGC 1173
     isCysLeuGlyAlaAlaGlyGlyLeuGluAlaIleAlaThrValLysAlaIleAsnThrGlyTrpLeuH

1174 ATCCCTCTATCAACCAATTTAACCCAGAACCAGCAGTGGACTTTGATACGGTCGCAAACGAGAAGAAGC 1242
     isProSerIleAsnGlnPheAsnProGluProAlaValAspPheAspThrValAlaAsnGluLysLysG
```

FIG. 11A - 3

```
1243 AGCATGAGGTGAATGTTGCCATATCAAACTCGTTTGGGTTCGGTGGACATAACTCAGTGGTCGCTTTCT 1311
     lnHisGluValAsnValAlaIleSerAsnSerPheGlyPheGlyGlyHisAsnSerValValAlaPheS

1312 CTGCCTTCAAACCCTGATTTCCTCAGACCCTTTAGATCCTCTGGTCCATCTGTTAGATCACCACCATCA 1380
     erAlaPheLysPro

1381 TCTTCTTCGCAGCTTCTTGGTTCACAAGTTGAGCGCTTTCTTCCTTTCAGCTTTTTGTTCTTATTGGTC 1449

1450 ATTGTTAATTTTTGCTCAACTCTTATTGGTCATTGAGGTGTAGAGAATCCAGATTTTGCTTCTACAATC 1518

1519 TGTGTACGGAATGTTGTATCTTTAGTTCGTTTTATGTTTGCCAAATTTTATAAAC 1573
```

FIG. 11A - 4

```
1   AACCACATTCGCCGTGGGGAAGCTGATATGATGATTGCTGGTGGAACCGAGGCTGCCATTATTCCTATT   69
    AsnHisIleArgArgGlyGluAlaAspMETMETIleAlaGlyGlyThrGluAlaAlaIleIleProIle

70  GGGTTGGGAGGTTTTGTTGCTTGCAGGGCGCTTTCGCAGAGGAATGATGACCCTAAAACCGCTTCGAGG  138
    GlyLeuGlyGlyPheValAlaCysArgAlaLeuSerGlnArgAsnAspAspProLysThrAlaSerArg

139 CCTTGGGATAAACAGAGAGATGGCTTTGTAATGGGTGAAGGAGCTGGTGTTCTGGTGATGGAAAGCTTG  207
    ProTrpAspLysGlnArgAspGlyPheValMETGlyGluGlyAlaGlyValLeuValMETGluSerLeu

208 GAACATGCGATGAAGCGTGGTGCGCCAATAGTAGCAGAGTATCTTGGAGGTGCTGTAAACTGTGATGCT  276
    GluHisAlaMETLysArgGlyAlaProIleValAlaGluTyrLeuGlyGlyAlaValAsnCysAspAla

277 CATCATATGACTGATCCAAGAGCTGACgGGCTTGGTGTCTCTTCATGCATTGAGAGCTGCCTTGAAGAT  345
    HisHisMETThrAspProArgAlaAspGlyLeuGlyValSerSerCysIleGluSerCysLeuGluAsp

346 GCTGGTGTTTCACCCGAGGAGGTAAATTACATCAATGCGCATGCAACTTCCACACTTGCTGGTGATCTT  414
    AlaGlyValSerProGluGluValAsnTyrIleAsnAlaHisAlaThrSerThrLeuAlaGlyAspLeu
```

FIG. 11B - 1

```
415 GCTGAGATTAATGCCATTAAAAAGGTATTCAAGAGCACTGCTGGGATCAAAATCAATGCCACCAAGTCT  483
    AlaGluIleAsnAlaIleLysLysValPheLysSerThrAlaGlyIleLysIleAsnAlaThrLysSer

484 ATGATAGGTCACTGCCTCGGTGCAGCTGGAGGTCTTGAAGCCATTGCGACTGTGAAGGCTATCAACACT  552
    METIleGlyHisCysLeuGlyAlaAlaGlyGlyLeuGluAlaIleAlaThrValLysAlaIleAsnThr

553 GGATGGCTTCATCCCTCAATCAACCAATTTaaCCCAGAACCAGCCGTGGACTTTGACACGGTCGCAAAC  621
    GlyTrpLeuHisProSerIleAsnGlnPheAsnProGluProAlaValAspPheAspThrValAlaAsn

622 GAGAAGAAGCAGCATGAGGTGAACGTTGCTATATCAAATTCGTTTGGGTTCGGTGGACACAACTCAGTT  690
    GluLysLysGlnHisGluValAsnValAlaIleSerAsnSerPheGlyPheGlyGlyHisAsnSerVal

691 GTCGCCTTCTCTGCCTTCAAACCCTGATTCCTTCAAGACCCTTTTGTATTTTCTTCTCCAACTATTACA  759
    ValAlaPheSerAlaPheLysPro

760 TCACCACCATCATCCATCAGGCATCATCTTCCTTGAGCTTCTTGGTTCCACGAGTTTGAGCTCTTTCTT  828
```

FIG. 11B - 2

```
829 TGGCGTTTTACGTTCCATTCAACATTGTTCTTATTGTTCATTGAGATTTCAAATTTTGCTTCTCAATCG  897

898 TAAGAAATGTTTGTATCTGTATCTGAGTTCGTTTCATATTTGTCTAATTTATAAACAGAACCA  966

967 ATAATCTTGTAGCAATGATGTTATTCAGAGTTCTCAATCTT 1007
```

FIG. 11B - 3

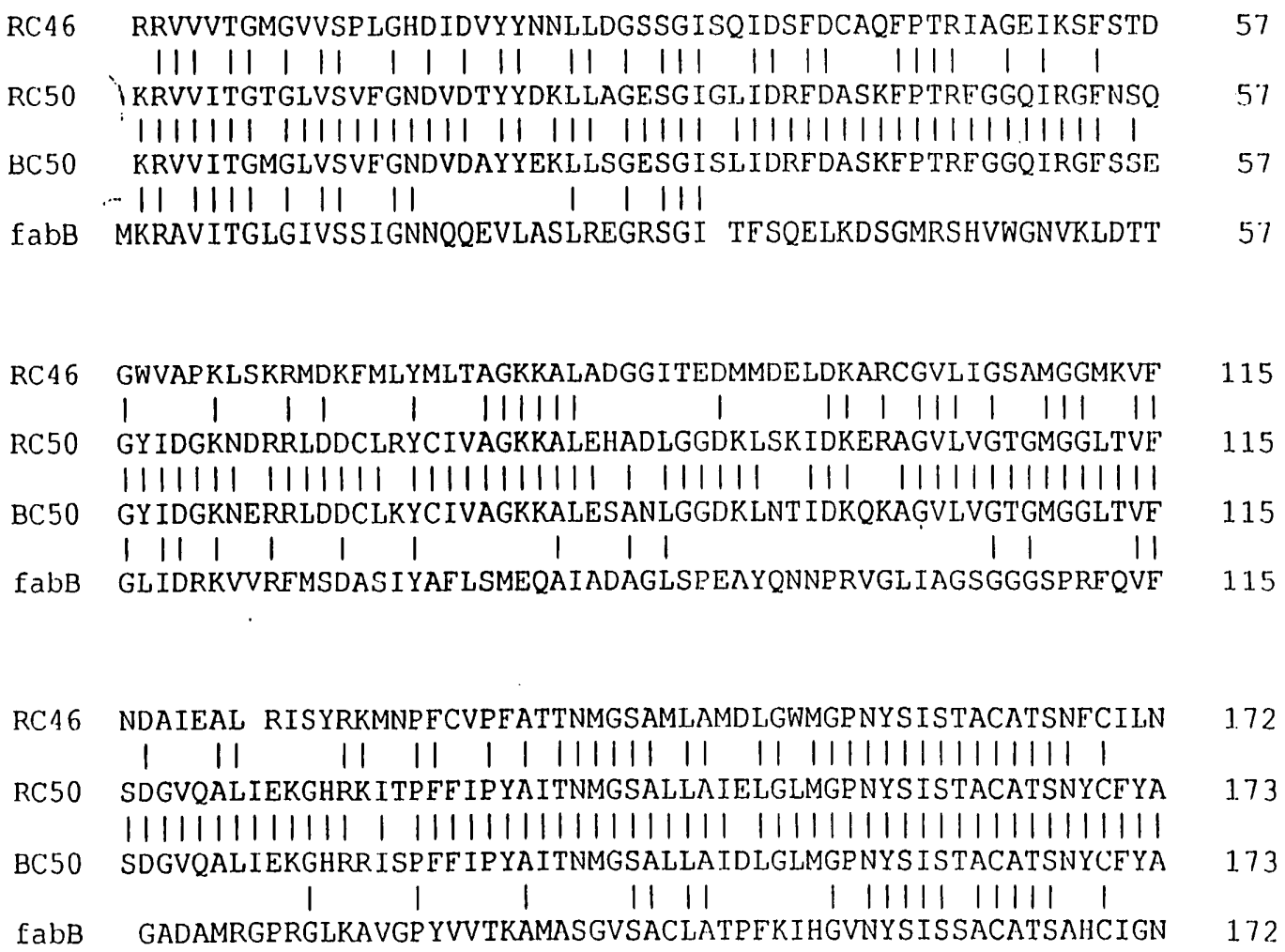
RC46 RRVVVTGMGVVSPLGHDIDVYYNNLLDGSSGISQIDSFDCAQFPTRIAGEIKSFSTD 57
RC50 KRVVITGTGLVSVFGNDVDTYYDKLLAGESGIGLIDRFDASKFPTRFGGQIRGFNSQ 57
BC50 KRVVITGMGLVSVFGNDVDAYYEKLLSGESGISLIDRFDASKFPTRFGGQIRGFSSE 57
fabB MKRAVITGLGIVSSIGNNQQEVLASLREGRSGI TFSQELKDSGMRSHVWGNVKLDTT 57
RC46 GWVAPKLSKRMDKFMLYMLTAGKKALADGGITEDMMDELDKARCGVLIGSAMGGMKVF 115
RC50 GYIDGKNDRRLDDCLRYCIVAGKKALEHADLGGDKLSKIDKERAGVLVGTGMGGLTVF 115
BC50 GYIDGKNERRLDDCLKYCIVAGKKALESANLGGDKLNTIDKQKAGVLVGTGMGGLTVF 115
fabB GLIDRKVVRFMSDASIYAFLSMEQAIADAGLSPEAYQNNPRVGLIAGSGGGSPRFQVF 115
RC46 NDAIEAL RISYRKMNPFCVPFATTNMGSAMLAMDLGWMGPNYSISTACATSNFCILN 172
RC50 SDGVQALIEKGHRKITPFFIPYAITNMGSALLAIELGLMGPNYSISTACATSNYCFYA 173
BC50 SDGVQALIEKGHRRISPFFIPYAITNMGSALLAIDLGLMGPNYSISTACATSNYCFYA 173
fabB GADAMRGPRGLKAVGPYVVTKAMASGVSACLATPFKIHGVNYSISSACATSAHCIGN 172

FIG. 12 - 1

```
RC46   AANHIIRGEADIMLCGGSDAAIIPIGLGGFVACRALS QRNDDPTKASRPWDMNRDGF   229
RC50   AANHIRRGEAELMIAGGTEAAIIPIGLGGFVACRALS QRNDDPQTASRPWDKDRDGF   230
BC50   AANHIRRGEADMMIAGGTEAAIIPIGLGGFVACRALS QRNDDPQTASRPWDKQRDGF   230
fabB   AVEQIQLGKQDIVFAGG GEELCWEMACEFDAMGALSTKYNDTPEKASRTYDAHRDGF   229

RC46   VMGEGAGVLLLEELEHAKKRGANIYAE FLGGSFTCDAYHMTEPRPDGVGVILCIEKA   286
RC50   VMGEGAGVLVMESLEHAMKRGAPIIAE YLGGAVNCDAYHMTDPRADGLGVSSCIERS   287
BC50   VMGEGAGVLVMESLEHAMKRGAPIVAE YLGGAVNCDAHHMTDPRADGLGVSSCIESC   287
fabB   VIAGGGGMVVVEELEHALARGAHIYAEIVGYGA     TSDGADMVAPSGEGAVRCMK   282

RC46   LARSGVSKEEVNYINAHATSTPAGDLKEYEALMRCFSQNPDLRVNSTKSMIGHLLGAA   344
RC50   LEDAGVSPEEVNYINAHATSTLAGDLAEINAIKKVFKNTSDIKINATKSMIGHCLGAA   345
BC50   LEDAGVSPEEVNYINAHATSTLAGDLAEINAIKKVFKSTSGIKINATKSMIGHCLGAA   345
fabB   MAMHGV DTPIDYLNSHGTSTPVGDVKELAAIREVF GDKSPAISATKAMTGHSLGAA   338
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FIG. 12 - 2

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RC46  GAVEAIATIQAIRTGWVHPNININLENPE EGVDTKVLVGPKKERLDIKVALSNSFGFGG   401
      |  ||||   || ||| || ||  |||   |        ||      || ||||||||
RC50  GGLEAIACVKAITTGWLHPTINQFNPE PSVEFDTVAN KKQQHEVNVAISNSFGFGG   401
      ||||||| |||| |||||| ||||||| | | |||||| ||| ||||||||||||||
BC50  GGLEAIATVKAINTGWLHPSINQFNPE PAVDFDTVANEKKQ HEVNVAISNSFGFGG   401
      |  |||        |   ||||                |     |     ||||||||
fabB  GVQEAIYSLLMLEHGFIAPSIN IEELDEQAAGLNIVTE TTDRELTTVMSNSFGFGG   394


RC46  HNSSIIFAPYK   412
      |||   |   |
RC50  HNSVVAFSAFKP  413
      ||||||||||||
BC50  HNSVVAFSAFKP  413
       |        |
fabB  TNATLVMRKLKD  406
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FIG. 12 - 3

I INTERNATIONAL SEARCH REPORT

International Applica No PCT/US91/05801

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 7/64; C12N 5/00; C07K 15/00; C07H 15/12
U.S.Cl.: 435/134,240.4; 530/ 377; 536/27

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched 7 Classification Symbols
U.S.	435/ 134, 240.4; 530/377 536/27; 800/ 205, Dig. 26, Dig. 69 935/60, 64, 67

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

USPTO Automated Patent System. Dialog files: Biotech, Patents
See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
Y	EP, A,0,255,377 (KRIDL et al) 03 February 1988, See entire document.	21-34,37-39 42-45,51-52

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 JANUARY 1992	28 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	P. Rhodes

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,394,443 (WEISSMAN et al) 19 July 1983, See entire document.	21-34, 37-39 42-45, 51-52
Y	Trends in Biotechnology, Volume 5, Issued 1987, Knauf, "The application of genetic engineering to oilseed crops", pages 40-47. See entire document.	1, 2, 15-16, 18, 21-34 37-39, 42-45 51-52
X	The Plant Cell, Volume 2, Issued April 1990, Napoli et al, "Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans", pages 279-289. See entire document.	21, 24, 28-33 38-39, 42, 44-45, 51-52
Y	Plant Molecular Biology: Proceedings NATO ASI series (Plenum Press), Volume 140, Issued 1987, Slabas et al, "Molecular structure of plant fatty acid synthesis enzymes", pages 265-277. See entire document.	1, 2, 15-16, 18, 21-34 37-39, 42-45 51-52
X	Carlsberg Research Communication, Volume 53, Issued 1988, Kauppinen et al, "B-ketoacyl-ACP synthase I of Escherichia coli: nucleotide sequence of the fabB gene and identification of the cerulenin binding residue", pages 357-370. See entire document.	21, 23, 25, 26 28-29, 31, 38
Y	Proceedings of the National Academy of Sciences USA, Volume 79, Issued October 1982, Shimakata et al, "Isolation and function of spinach leaf B-ketoacyl-[acy-carrier-protein] synthases", pages 5808-5812. See entire document.	21-22, 24-34 37-39, 42-45 51-52
Y	Biochimica et Biophysica Acta, Volume 1002, Issued March 1989, MacKintosh et al, "A new assay procedure to study the induction of B-ketoacy-ACP synthase I and II, and the complete purification of B-Ketoacy-ACP synthase I from developing seeds of oilseed rape (Brassica napus)", pages 114-124. See entire document.	1-2, 15-16 18, 21, 23-34 37-39, 42-45 51-52

ATTACHMENT TO FORM PCT/ISA/210:
Part VI. Observations (continued, page 3 of 3)

IX. Claims 53, 57, 62, 65, and 67, drawn to a method of modifying fatty acid composition of plants, classified in Class 435, subclass 172.1, for example.

Note that the following are independent and distinct species pertinent to the invention of Group IX where t) is the first species and will be searched with claims 53, 57, 62, 65, and 67 in the event that no other fees are paid. Note that a search of any other additional species within Group IX requires payment of additional fees.

t) where the encoding sequence is an antisense sequence (claims 54-55 and 63);

u) where the encoding sequence is a sense sequence (claims 56 and 64).

X. Claims 58-61 and 66, drawn to plant cells and seeds having a modified fatty acid composition, classified in Classes 435 and 800, subclasses 240.4 and 250, respectively, for example.

XI. Claims 68-69, drawn to oil, classified in Class 426, subclass 601, for example.

ATTACHMENT TO FORM PCT/ISA/210:
Part VI. Observations (continued, page 2 of 3)

VI. Claims 21, 24-34, 37-39, 42-45, and 51-52 drawn to a recombinant DNA construct encoding a plant synthase and the transgenic plant cells containing same, classified in Classes 435 and 536, subclasses 240.4 and 27, respectively, for example.

Note that the following are independent and distinct species pertinent to the invention of Group VI where h) is the first species and will be searched with claims 21, 24-34, 37-39, 42-45, and 51-52 in the event that no other fees are paid. Note that a search of any other additional species within Group VI requires payment of additional fees.

h) where the encoded synthase is factor A (claim 22);

i) where the encoded synthase is factor B (claim 23);

j) where the encoded synthase is required for longer chain fatty acids (claims 35 and 46);

k) where the encoded synthase is required for shorter chain fatty acids (claims 36 and 47).

VII. Claim 40, drawn to a second transgenic plant cell comprised of a first DNA construct and a second DNA construct, classified in Class 435, subclass 240.4, for example.

Note that the following are independent and distinct species pertinent to the invention of Group VII where l) is the first species and will be searched with claim 40 in the event that no other fees are paid. Note that a search of any other additional species within Group VII requires payment of additional fees.

l) where the first component is factor A (claim 22) and the second component is desaturase (claim 41);

m) where the first component is factor A (claim 22) and the second component is thioesterase (claim 41);

n) where the first component is factor B (claim 23) and the second component is desaturase (claim 41);

o) where the first component is factor B (claim 23) and the second component is thioesterase (claim 41);

p) where the first component is required for longer chain fatty acids (claim 35) and the second component is desaturase (claim 41);

q) where the first component is required for longer chain fatty acids (claims 35) and the second component is thioesterase (claim 41);

r) where the first component is required for shorter chain fatty acids (claims 36) and the second component is desaturase (claim 41);

s) where the first component is required for shorter chain fatty acids (claims 36) and the second component is thioesterase (claim 41);

VIII. Claims 48-50, drawn to a method of making protein, classified in Class 435, subclass 69.1, for example.

ATTACHMENT TO FORM PCT/ISA/210:
Part VI. Observations Where Unity of Invention is Lacking

These claims present a plurality of mutually exclusive independent inventions as follows:

I. Claims 1-2 and 15-16, drawn to a first protein product and process of use to make short chain fatty acids, classified in Classes 435 and 530, subclasses 134 and 377, respectively, for example.

Note that the following are independent and distinct species pertinent to the invention of Group I where a) is the first species and will be searched with claims 1-2 and 15-16 in the event that no other fees are paid. Note that a search of any other additional species within Group I requires payment of additional fees.

a) an in vitro process (claim 18);
b) an in vivo process (claims 19-20);

II. Claims 3-4, drawn to a second protein product, classified in Class 530, subclass 377, for example.

III. Claim 6, drawn to a third protein product, classified in Class 350, subclass 377, for example.

Note that the following are independent and distinct species pertinent to the invention of Group III where c) is the first species and will be searched with claim 6 in the event that no other fees are paid. Note that a search of any other additional species within Group III requires payment of additional fees.

c) the product where the preparation is from a first major fraction (claims 7-8);
d) the product where the preparation is from a later major fraction (claim 9).

IV. Claims 13-14, drawn to a nucleic acid sequence, classified in Class 536, subclass 27, for example.

Note that the following are independent and distinct species pertinent to the invention of Group IV where e) is the first species and will be searched with claims 13-14 in the event that no other fees are paid. Note that a search of any other additional species within Group IV requires payment of additional fees.

e) the product where the sequence encodes a 50 kd protein of the first major fraction (claim 10);
f) the product where the sequence encodes a 46 kd protein of the first major fraction (claim 11);
g) the product where the sequence encodes a 50 kd protein of the later major fraction (claim 12).

V. Claim 17, drawn to a process of use for the product of Group II to make long chain fatty acids, classified in Class 435, subclass 134, for example.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

see attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

1-2, 15-16, 18, 21-34, 37-39, 42-45, 51-52

as instructed in the response received 16 December 1991.

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT TO FORM PCT/ISA/210
Part II. FIELDS SEARCHED TERMS

ketoacyl; synthase or synthetase; fatty acid; synthesis or biosynthesis; plant? or seed; spinach or brassica or soybean or ricinus